MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF VARIABILITY IN *IN VITRO* DERIVED SEEDLINGS OF VANILLA (*Vanilla planifolia* Andrews)

By

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THESIS

submitted in partial fulfilment of the requirement for the degree of

Master of Science in Horticulture

Faculty of Agriculture
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2005

DECLARATION

I hereby declare that this thesis entitled "**Morphological and molecular characterization of variability in *in vitro* derived seedlings of vanilla (*Vanilla planifolia* Andrews)**" is a bona-fide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled "**Morphological and molecular characterization of variability in *in vitro* derived seedlings of vanilla (*Vanilla planifolia* Andrews)**" is a record of research work done independently by Ms. **Hena, K.K.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Dedicated to beloved
Achan and Amma

Introduction

INTRODUCTION

Vanilla is the only orchid spice and the main source for natural vanillin. The worldwide interest for factoring in natural products in the health agenda has given a new fillip to vanilla. No one can sweep under carpet the perils associated with synthetic vanillin.

Among the food flavours used all over the world, vanillin owns a prime position. Vanilla essence (vanillin) extracted from the cured beans of vanilla is largely used for flavouring ice creams, chocolates, bakery products, puddings, liquors and perfumes. The demand for vanilla flavour is on the rise. Nowadays vanilla essence is also used for flavouring cashewnuts, ginger products, fruits etc.

Native of Mexico, vanilla is now cultivated in many countries like Madagascar, Mexico, Comoro and Reunion, Indonesia, Tahiti, Seychelles, Malaysia, Sri Lanka, Latin America, Uganda, Tongo etc. India is also emerging as a major producer of vanilla. The history of introduction of vanilla to India is rather obscure. It is believed that vanilla was introduced to India about 200 years ago for planting in the 'Spices Garden' at Kourtallam (Tamil Nadu) owned by British East India Company. In India, the vanilla of commerce is *Vanilla planifolia* Andrews and this is the predominant species cultivated all over the world. The other cultivated species are *Vanilla pompona* Schiede and *Vanilla tahitensis* Moore. A few more wild species of vanilla that occur in India are *V. vatsalae*, *V. walkeriae*, *V. wightiana*, *V. andamanica* and *V. pilifera*. *Vanilla vatsalae*, *V. walkeriae* and *V. wightiana* are leaf less forms, they have poor quality.

Being a climbing orchid, vanilla has to be trailed on suitable standards. It can be grown as an intercrop in coconut and arecanut plantations and as an alternate crop in cardamom and coffee plantations. Flowering commences in the third year of planting, usually. The flowers are to be artificially pollinated, as the natural pollinators are not occurring in India. Vanilla beans mature within seven to nine months of pollination. Timely scientific processing of harvested beans is very essential for premium quality of vanilla. Vanillin content of properly cured beans will not be less

than two percent. A properly maintained vanillery yields about 300 to 600 kg cured beans per hectare.

Variability is the essence of any crop improvement programme. Being vegetatively propagated, the existing variability in vanilla is very low. Natural seed germination is limited and the crop has a very narrow genetic base. However, being a cross-pollinated crop, there is ample scope for creating variability through seedling propagation. *In vitro* techniques could be successfully utilized for induction of variability in vanilla. The Centre for Plant Biotechnology and Molecular Biology (CPBMB) at College of Horticulture, Vellanikkara is maintaining vanilla plants derived *in vitro* through seed culture. Preliminary observations have revealed some phenotypic differences among those seedlings. The discovery of isozyme markers and polymerase chain reaction has now made the genetic finger printing easier in crop plants. Many plant genomes have been characterized using such techniques like RAPD (Random Amplified Polymorphic DNA) and RFLP (Restriction Fragment Length Polymorphism). Results of RAPD analysis are independent of environmental influences, tissue type etc. and provide greater resolution than the other techniques. The procedure is faster and easier than other molecular analyses.

Characterization of the vanilla plants using morphological and molecular markers like isozyme and RAPD is a valuable tool for identification of the genetic variability. The present study therefore attempts to characterize the variability in the field established vanilla (*Vanilla planifolia* Andrews) plants derived from *in vitro* seed culture. The characterization is done using;

1. Morphological markers
2. Biochemical markers:
    i. Esterase
    ii. Peroxidase
3. Molecular markers:
    i. DNA isolation
    ii. RAPD analysis.

Review of Literature

2. REVIEW OF LITERATURE

Vanilla planifolia Andrews the only spicy orchid of the tropics is valued for its cured fragrant beans which make it one of the most expensive spices second only to saffron. It is native to the humid tropical rain forests of South Eastern Mexico, Central America, the West Indies and northern part of South America. *Vanilla* L was derived from the Spanish *vainilla*, a diminutive of *vaina*, a pod. Its specific epithet, *planifolia* refers to the broad, flat leaf of the plant. Vanilla is the most important spice from the west.

India is one of the potential countries for vanilla cultivation and it can be grown as an intercrop in coconut and arecanut plantations and as an alternate crop in cardamom and coffee plantations. In recent times its potentialities have been realized and vanilla cultivation is recognized as one of the attractive proposition in agriculture, which yield considerable income in a short span of time. Vanilla cultivation in India is mainly concentrated in certain areas in Kerala, Tamil Nadu and Karnataka.

2.1 BOTANY OF VANILLA

Vanilla planifolia Andrews belongs to the family Orchidaceae, an advanced group of monocotyledons. The family is the largest one of the flowering plants with 700 genera and 20,000 species. One hundred and ten species of vanilla are reported consisting of terrestrial, climbing, epiphytic and saprophytic species.

Vanilla planifolia Andrews is the most commonly cultivated species of 'Vanilla of Commerce' apart from *Vanilla pompona* Schiede (West Indian vanilla) and *Vanilla tahitensis* J.W. Moore (Tahitian vanilla). The basic chromosome number for the genus *Vanilla* is x =16 and *Vanilla planifolia* is a diploid with 2n =32. In countries where vanilla has been introduced, variability is likely to be highly limited. The material is propagated vegetatively, hence of clonal origin (Madhusoodanan *et al.*, 2003).

Being an orchid, the propagation is mainly through vegetative means. Thus there is a lack of natural variability in vanilla. Efforts are being made to produce

seedlings through *in vitro* techniques and thereby inducing variability. The present investigations on 'Morphological and molecular characterization of variability in *in vitro* derived seedlings of vanilla (*Vanilla planifolia* Andrews)'are aimed at detecting the variability in field established *in vitro* derived vanilla plants using morphological and molecular markers.

2.2 *IN VITRO* SEED CULTURE OF VANILLA

Vanilla pods attain full maturity in about 10 to 12 months and green pods of 5 to 7 months maturity were found ideal for *in vitro* seed germination. Divakaran *et al.* (1996) have reported MS medium as the best for *in vitro* ovule culture of vanilla. *In vitro* techniques could be successfully utilized for induction of variability in vanilla. The Centre for Plant Biotechnology and Molecular Biology (CPBMB) at College of Horticulture, Vellanikkara is maintaining vanilla plants derived through *in vitro* seed culture. Divakaran *et al.* (1996) studied various morphological parameters like leaf size, internodal length, growth rate and isozyme pattern in vanilla seedlings raised through ovule culture and they observed genetic variability in the progenies. Variability has been observed in the seedlings especially in their leaf morphology and phyllotaxy (Mary *et al.*, 1999).

2.3 MORPHOLOGICAL MARKERS

The review focuses on the field performance and extent of variation in phenotypic characters in *in vitro* derived plants.

2.3.1 Variation in phenotypic characters in *in vitro* derived plants

2.3.1.1 Spices

In order to develop a model for determination of vine length based on biometrical observations, 100 plants of *Vanilla planifolia* Andrews were randomly selected and observations were made on vegetative characteristics. Number of nodes, number of leaves and internodal length have been reported to be significantly and positively correlated with vine length. A multiple regression equation was derived which had 82.5 per cent precision (Shankaran *et al.*, 1994).

Sudharshan *et al.* (1997) evaluated mircropropagated cardamom plants for growth and yield. Tissue culture derived clones showed variations in the type of panicle, capsule shape and size. The overall variability in tissue cultured plants was observed as 4.5 per cent as against 3 per cent in open pollinated seedling progenies. Chandrappa *et al.* (1997) evaluated tissue cultured promising cardamom selections for their yield performance for three years. The lines TC5, TC6 and TC7 were found promising as compared to the other lines. These three selections also differed among themselves for yield and yield attributes. Clonal crop of the two ruling varieties viz. Mudigere 2 yielded significantly low compared to TC5 but was on par with other seven tissue cultured selections.

Nazeem *et al.* (1998) tried to induce variability in ginger through indirect organogenesis and *in vitro* mutagenesis. Calli were induced from different explants of ginger var. Maran. The regenerants from irradiated culture were hardened and planted out. The plantlets were maintained and evaluated in the field for two seasons. They observed considerable variations for growth parameters and abiotic stress tolerance.

Sanchu (2000) performed the variability analysis in calliclones of black pepper (*Piper nigrum* L.). She measured the extent of variability in the field planted calliclones of black pepper (cv. Cheriakanyakkadan) for yield, quality and reaction to *Phytopthora* foot rot disease.

2.3.1.2 Fruit crops

Vuylsteka *et al.* (1996) identified four types of morphologically distinct somaclonal variants in a population of False Horn plantain (*Musa* spp. AAB group) produced by shoot tip culture. Field evaluation of these variants showed that three of the four variants were horticulturally inferior due to inflorescence degeneration and abnormal foliage. In contrast, the variations reported in the Cavendish bananas propagated by apical meristem were low as reported by DeGomez and DeGarcia (1997). Grillo *et al.* (1999) reported the use of banana somaclonal variants for ornamental purpose. Variation has been found for plant height, leaf shape and color, pseudostem morphology and color, reproductive organ morphology and sucker emission rate.

Martin *et al.* (1994) observed somaclonal variation in field planted *in vitro* produced pineapple clones. They categorized the clones as dwarf, variegated, thorny leaved and pigmented as compared to the control plants.

Clones of two cultivars of datepalm viz., Thoory and Zadhi, of date palm produced through *in vitro* culture were field analyzed for two successive seasons to evaluate the flowering behavior and fruit set by A1-Ghamdi (1996). He observed significant difference between the cultivars in flowering date, flowering duration and number of flowers produced per tree per week, number of bunches, spikes, fruit drop, total fruits per tree and percentage fruit set.

McPheeters and Skirvin (1989) compared the regenerants produced from shoot tip culture of black berry for different vegetative and reproductive traits and observed variation in growth habit, flower number and fertility. Norton and Skrivin (1997) evaluated the stability of the thornless character in thornless evergreen somaclones of black berry after seven years of field planting. They observed thornlessness as a stable variation and thornless character was directly related to dwarfism.

2.3.1.3 Solanaceous vegetables

Thomas (1981) reported large amount of variation in growth and leaf morphology in plants regenerated from stem tip culture derived protoplast of the tetraploid British potato cultivar Maris Bard. Thomson (1987) observed large difference in total harvested yield among the somaclones derived from protoplast cultures of cultivars Feltwell and Marispiper. Six out of 197 of the cultivar Feltwell and two out of 229 of Marispiper somaclones out yielded their parents. Carrasco *et al.* (1998) reported significant variation for seven of the eight morphological traits studied in potato protoclones evaluated in a field experiment.

Zagorska *et al.* (1986) reported variability in morphological and cytological traits and pollen fertility in regenerants obtained from tissue cultures of tomato leaves and flower buds. Self compatible forms from initially completely self incompatible

Somasundar and Gostimsky (1992) reported high frequency somaclonal variation for agronomically important traits such as early flowering and orange fruit color in tomato.

2.3.1.4 Ornamental plants

Ohishi and Sakurai (1988) observed morphological changes in chrysanthemum somaclones derived from petal tissue. High incidence of mutations like increase in disc florets or decrease in ray florets were observed in the somaclones and increase in disc florets was linked to male sterility. Other variations noted in the population were in petal color, flower shape and size and leaf shape.

Laneri (1990) reported somaclonal variations in plantlets derived from *in vitro* cultured immature flower buds of cymbidium. The somaclones produced racemes with a slightly longer labellum and more pointed, yellow brown tepals.

Begonia x elator plantlets regenerated from leaf disc callus showed differences in flower morphology, flower size, plant height, plant morphology and number of flowers per plant (Jain, 1993). Similar somaclonal variations were reported in *Saintpaulia ionantha* (Jain, 1993, El-Mardi *et al.*, 1993) and *Zinnia marylandica* (Stieve and Stimart, 1992). Arena *et al.* (1993) reported that plants regenerated from callus in *Rosa hybrida* (cv. Meirutral) exhibited variation in number, color and shape of petals, growth habit and height.

In Rudbeckia, plants regenerated through callus cultures exhibited variation in terms of flower shape, number of ray florets/flower, flower color, polyploidy and aneuploidy (Khilbas, 1995).

Buiatti *et al.* (1996) assessed the field performance of plantlets derived from petals, mericlones and cuttings of carnation (cv. Corrida) and obtained higher heritability for plant height, flower number and flowering date.

2.3.1.5 Aromatic plants

CIMAP (1992) released two improved somaclones of *Mentha arvensis* SC-93 and SC-179 from multiple shoot cultures of CIMAP hybrid-77. These soma clones were characterized by higher herbage yield, better regeneration of foliage after the first cut, better harvesting index in the first cut, lower distillation costs and increased tolerance to high moisture regimes. Han *et al.* (1998) reported greater genetic variation in regenerants from young stem segments of pepper mint var. 73-8 for fresh weight per plant, flowering date, stem and branch characters.

Mathur *et al.* (1988) evaluated the plants regenerated from leaf sheath derived callus cultures of (*Cymbopogon winterianus* Jowitt) variety Jorhat. Out of 500 plants, 250 showed extensive somaclonal variation for six or seven agronomic traits viz., herbage yield, tiller number, diameter of the tiller, area of longest leaf and fresh and dry weight ratio. Patnaik *et al.* (1999) reported somaclonal variation in cell suspension culture regenerants of *Cymbopogon martini* (Roxb.) wats. var. motia for plant height, yield and tiller number.

2.3.1.6 Field crops

Ivanov *et al.* (1998) evaluated somaclones (R_3 and R_4 generations) regenerated from five winter wheat genotypes for plant height, top internode length, spike length, number of seeds per spike and hundred seed weight. They concluded that plant height and top internode length of all somaclones derived from cv. Charodeika and Pliska decreased while spike length of Moulina increased compared to their parents. Villareal *et al.* (1999) compared tissue culture derived lines (TCDL) of spring wheat cv. Pavon with the parent cultivar and reported that TCDL possessed greater number of grains m^{-2}, spikes m^{-2} and grains $spike^{-1}$,early flowering and dwarf habit.

Shen *et al.* (1993) isolated desirable somaclonal variants from IR-26 showing increased 1000-grain weight, grains per panicle and grain weight per plant. In a field experiment conducted to examine the variability in eight rice somaclones, Abbasi *et al.* (1999) reported a significant reduction in plant height and days to flowering and increase in kernel length and recovery percentage. They selected somaclone TF4 having stiff stems, earliness in flowering, semidwarf habit with high fertility and greater yield potential than parent. They noted that grains of somaclones were fine, slender and long.

Maralappanavan *et al.* (1995) evaluated somaclonal variation for quantitative characters in plants regenerated from well-established callus of two popular rabi sorghum var. M-35-1 and A-1. They observed that nine families out of seventy six showed morphological variation for characters like chlorophyll, leaf arrangement and mid rib structure in M-35-1 and three out of 30 showed variations in branching pattern and male sterility in A-1 lines.

Dhumale *et al.* (1994) reported variation in yield and in number of internodes per plant in the callus regenerants of cv. COC 671 of sugarcane. Taghian and Fahmy (1998) evaluated 90 somaclones regenerated from tissue culture of sugarcane variety C-310 and their donor parent. They found significant differences among the somaclones than the donor parent and higher heritability was found for stalk length (98%), stalk number (73%), yield (73%) and stalk volume (72%).

2.4 MOLECULAR MARKERS

In the past several years genetic studies have led to the establishment of several types of molecular markers which include biochemical markers like isozyme markers and DNA markers. Protein and DNA markers are together called molecular markers. Molecular markers based on genomic DNA have been reported to have great significance in finger printing individuals and in genetics and plant breeding studies.

2.4.1 Isozyme markers

This is one of the biochemical markers most widely exploited is protein characteristics. Proteins extracted from different genotypes or tissue sources can be compared by the techniques of electrophoresis. The spectrum of proteins present in the extract can be separated and compared on the basis of size (molecular weight), charge and conformation and are visualized by staining. The discovery of isozymes by Hunter and Market in 1957 has played an essential role in many branches of biology like taxonomy, host pathogen interaction analysis and evolutionary studies. Today it has become the most widely recognized link between the organism and molecular approach to science. Isozymes are different forms of the same enzymes having identical or similar functions and present in the same individual (Market and Moller, 1959). Utilization of isozyme profiles has proved valuable for cultivar identification in many agricultural plants. Correlation between isozyme variation and the geographic origin of plant accessions is also possible (Soltis *et al.*, 1983; Vallejos, 1983 and Glaszmann, 1987). Isozyme variations are used as a powerful tool to compliment conventional biochemical and genetic studies (Yndgard and Hoskuldson, 1989).

2.4.1.1 Spice crops

Kochhar *et al.* (1989) noted characteristic taxonomic markers for six varieties in betel vine by peroxidase pattern study. In ginger (*Zingiber officinalis*) twenty-eight cultivars were compared for peroxidase isozyme patterns by fuzzy cluster analysis in Fujian. The cultivars differed in isozyme pattern activity and intensity. They were divided into three types viz., da-fei-jiang, huang and zhu-zi-zang (He *et al.*, 1995).

Sebastian (1995) reported that the ideal part for analysis of peroxidase was root or mature leaf and immature leaf for glutamate oxaloacetate transaminase (GOT) and esterase in black pepper (*Piper nigrum* L.). He also reported considerable variation at interspecific and intraspecific level by carrying out isozyme analysis in 26 varieties and 11 species of pepper. They were grouped based on peroxidase, esterase and glutamate oxaloacetate transaminase (GOT) banding pattern.

Seedling progenies of turmeric (*Curcuma longa*) showed maximum similarity and differed distinctly from the clonally propagated accessions through isozyme analysis. Shamina *et al.* (1998) collected fifteen accessions of *Curcuma longa* from different geographical areas in India along with a few seedling progenies and studied them for variation based on polymorphism on isozyme.

2.4.1.2 Isozyme markers in other horticultural crops

Bashan *et al.* (1987) studied the relation of enzymes and resistance against *Pseudomonas syringae* pv. tomato which revealed presence of four dibased peroxidase isozymes in extracts from diseased plants, while only one was present in healthy plants of tomato.

Isozyme banding pattern has been reported as genetic marker in peach (*Prunus persica* L.) It was investigated using starch gel electrophoresis. Leaf samples were taken from both juvenile and mature plants. A survey of 38-enzyme activity stain and five electrophoretic buffer systems were conducted. Only 12 staining systems produced well-resolved banding patterns; of these, nine were monomorphic among all genotypes surveyed and showed some variation (Durham *et al.*, 1987).

Isozyme variation was studied at 15 loci resolved from eight enzyme systems for 15 varieties of Cherimoya (*Annona cherimola*) and one variety of Atemoya (*Annona cherimola* x *Annona squamosa*). Each of the cultivars of Cherimoya and Atemoya showed distinct patterns (Ellstrand and Lee, 1987).

Polyphenol oxidase isozymes were studied by from healthy roots of tomato variety Pusa Ruby infected by *Meloidogyne incognita*. The absence of a band with Rm value of 0.520 in healthy or apparently healthy tissue was reported (Ganguly and Dasgupta, 1988).

Peroxidase activity was used as a biochemical marker for the resistance of musk melon (*Cucumis melo*) to *Pseudopernospora cubensis*. The activity of the

infected plants was higher than that of the uninfected plants. There were about 257 plants including cultivars, breeding lines and crosses of susceptible and resistant plants, which were used to predict the resistance and susceptibility (Reuveni *et al.* 1991).

Isozyme was used as genetic marker to characterize seven Spanish Cherimoya (*Annona cherimola*) cultivars. Fifteen enzyme systems were analyzed. Two cultivars only had identical banding pattern for all enzymes tested (Pascual *et al.*, 1993).

Based on the peroxidase isozyme pattern obtained in 41 *Malus* accessions the systematic positions of the 41 genotypes in the genus *Malus* were examined (Li *et al.*, 1995).

Esterase isoenzyme could be used to discriminate lime (*Citrus aurantifolia*) cultivars and peroxidase isoenzyme showed no difference among genotypes (Satrabhandhu *et al.*, 1996).

Isozyme markers were used to identify several cultivars of Purple loose strife (*Lathyrum* sp.) and interspecific hybrids. There are two zones of activity for phosphoglucomutase (PGR), phosphoglucoisomerase (PGI), and two zones for malate dehydrogenase (MDH) in Purple loosestrife (Strefeler *et al.*, 1996).

The intra and inter population variation in the reaction of adaptation of individuals of *Elytrgia repens* collected at different altitudes in eastern Germany showed variability for esterase and peroxidase isozyme pattern (Guttel and Hartenstein, 1996).

Peroxidase and esterase isozyme banding patterns were used as genetic markers of resistance for mosaic virus in pumpkin (Kuriakose, 1998).

Bose (1999) reported that peroxidase and polyphenol oxidase isozyme banding patterns could be used as genetic markers of resistance in tomato for bacterial wilt disease.

Somsri (1999) used isozyme variation to differentiate the sex in papaya. Among 36 isozymes studied about nine isozymes were useful to differentiate the sex in papaya. Of these, peroxidase, leucine amino peptidase (LAP) and esterase were able to distinguish male from female plants in Australian cultivar Richter and peroxidase and phosphoglucoisomerase (PGI) were able to distinguish hermaphrodite from female plants in the Hawaiian cultivar Sunset.

Four isozymatic systems were used to detect the genetic diversity in lemon and lemon like citrus cultivars. Leaf tissues were used to analyze the isozymes of *Citrus limon, C. aurantifolia, C.latifolia, C.mayeri* and two cultivars of local selections. The isozymes malate dehydrogenase (MDH), glutamate oxaloacetate transaminase (GOT), tetrazolium oxidase (TO) and esterase were analysed. The GOT enzyme difference was found adequate for the distinction at the species level. Esterase zymograms were very much useful as a diagnostic tool for cultivar identification in view of the extensive polymorphism of this enzyme.

2.4.1.3 Isozyme markers in field crops

The usefulness of esterase banding patterns was analysed for identifying the cultivars of cassava using the extracts of the viable roots of the cultivars (Hussain *et al.*, 1987).

Liu *et al.* (1988) reported that shoots and upper leaves of smut resistant millet cultivars showed more number of peroxidase and polyphenol oxidase bands compared to susceptible cultivars. They suggested the possible use of above observations as a marker for selecting smut resistant cultivar in maize.

The levels of total phenol, polyphenol oxidase and peroxidase in the leaves of Alternaria leaf blight resistant and susceptible cultivars of *Brassica* spp. were studied .It was reported that there was an increased level of total phenol and more

number of bands for polyphenol oxidase in resistant cultivars (Gupta *et al.*, 1995). Fei *et al.* (1997) reported that soybean resistant cultivars had four additional bands for peroxidase isozymes than susceptible cultivars.

The susceptible and resistant interactions between cassava (*Manihot esculenta*) and *Xanthomonas axonopodis* pv. *manihotis* and *Xanthomonas cassavae* were understood by using the peroxidase activity. It was found that cell wall bound peroxidase activity in the resistant interactions were two fold higher than that of the control or susceptible interactions which may be related to lignin deposition (Pereira *et al.*, 2000).

Six isozyme systems were used to assess the genetic relationship among *Saccharum* species clones. Species affinities as revealed through den drogram constructed based on phylogenetic analysis using parsimony indicated the closeness between *S. barberi* and *S. sinense* and between *S. officinarum* and *S. robustum* clones (Hemaprabha and Rangasamy, 2001).

2.4.2 DNA markers

Although the isozyme markers have proved to be very useful, they do not provide a complete picture of genes. All the characters may not be expressed uniformly as their expression may be related to environmental or physiological factors. The discovery of Polymerase Chain Reaction has now made the DNA fingerprinting easier in crop plants. Many plant genomes have been characterized using such techniques.

More recently, methods to analyse the genome at the DNA level have been developed. The advantages of these methods are greater resolution and uniformty of DNA in all cells. Several DNA markers have been developed in the recent years based on the extensive variation at DNA level among natural populations. Most important among these markers are RFLP and RAPD. Results of RAPD analysis are independent of environmental influences, tissue type etc. and provide greater resolution than the other techniques. The procedure is faster and easier than other

molecular analyses. The standardization of RAPD analysis would greatly help in the characterization of germplasm accessions.

The detection and exploitation of naturally occurring DNA sequence polymorphism represents one of the most significant recent developments in molecular biology. The technical complexity of performing RFLP analysis coupled with the wide spread use of short lived radioisotopes in detection method has prompted a debate on whether the routine and large scale application of RFLPs in crop improvement programmes is feasible. Since its development, PCR (Saiki *et al.*, 1988) has revolutionized many standard molecular biological techniques with modification of the original procedure designed to suit a range of needs. One such variation generates a specific class of molecular markers termed RAPD.

DNA amplification with random sequence primers is a highly sensitive method for discovering polymorphism randomly distributed through out the genome. The amplified products are called RAPDs (Williams *et al.*, 1990).

Variation in the DNA sequence is observed as differences in the ability to bind to short oligonucleotide primers used in the PCR. These primers are synthetically produced, random DNA sequences, approximately 10 nucleotides in length, with 50-60 per cent GC content (Welsh and McClelland, 1990).

The RAPD assay has been used for genetic finger printing in crop plants like rice (Fukuoka *et al.*, 1992), brassica species (Demeke *et al.*, 1992). RAPD fingerprinting in 20 micro propagated plants and the mother plant in *Piper longum* has been reported by Parani *et al.* (1997). The RAPD fragments were scored for the presence or absence of bands to evaluate Jaccard's similarity index. Further analysis of the data showed 18 micro propagated plants forming a major cluster along with the mother plant. The other two plants could be regarded as somaclonal variants as they have shown 80 percent similarity to the other plants and other micro propagated plants.

Walther *et al.* (1997) described the possibility of early detection of *in vitro* mutants using RAPD analysis. Four different types of somaclonal variants were identified and characterized in banana plants generated by meristem culture.

Babu (1997) optimized the protocol for RAPD analysis in black pepper. The primers identified for varietal screening and the RAPD profile developed for the five important varieties could be utilized for finger printing of other varieties. The results also ensured the genetic stability and clonal fidelity of the tissue culture plants and the suitability of tissue culture protocol for commercialization.

Recent studies conducted at Indian Institute of Spices Research, Calicut indicated that the progenies of vanilla are more similar to their parent *Vanilla planifolia* and to each other. There was reasonable degree of variability within the selfed progenies of vanilla. RAPD polymorphism observed indicated that *Vanilla planifolia* and *Vanilla aphylla* are distinct. The profiles coupled with morphological characters indicated that VH1, VH4 and VH5 are true interspecific hybrids between *Vanilla planifolia* and *Vanilla aphylla* as they are approximately equi-distant from the parents (IISR, 2000).

Materials and Methods

3. MATERIALS AND METHODS

The study on morphological and molecular characterization of variability in *in vitro* derived seedlings of vanilla (*Vanilla planifolia* Andrews) was conducted during the period 2001-2003 at the College of Horticulture, Vellanikkara.

3.1 MATERIALS

The vanilla accessions studied included *in vitro* derived clones regenerated from half mature pods of vanilla. Such *in vitro* seedlings derived clones originated from same pod, same seed and those from different pods were included in the study. The *in vitro* derived two year old plants already established in the field and maintained in Centre for Plant Biotechnology and Molecular Biology, College of Horticulture were screened to detect variability(Plate 1). Altogether 360 such plants were observed to detect variability through morphological markers. Details of clones studied are provided in Annexure 1. From this, 20 plants exhibiting high phenotypic variation were subjected to characterization using biochemical and molecular markers. The details of clones selected are provided in Annexure 2.

3.2 METHODS

3.2.1 Variability in morphological characters

The 72 vanilla accessions were critically observed for morphological characters. Totally 360 plants were observed i.e., five plants from each accession. Observations on different characters (as detailed below) were recorded for each clone separately and the data were interpreted statistically.

3.2.1.1 Vegetative characters

The vegetative characters recorded were leaf characters like, leaf length, leaf width, leaf area, number of leaves, leaf phyllotaxy and leaf shape; stem characters like internodal length and growth rate; epiphytic root characters like number and origin with the following specifications. The leaf characters were recorded for those that were fully expanded (3^{rd} leaf to 5^{th} leaf form the tip).

Plate 1. Variability in growth pattern of 2 year old seedling derived clones of vanilla

Character	Method adopted
Leaf length	Measured from tip of the leaf to the base of the leaf lamina (cm)
Leaf width	Measured at the widest portion on the leaf lamina (cm)
Leaf shape	Observed and recorded for all the clones
Leaf area	Leaf area was computed as follows: Leaf area = -62.246 + 3.376 l + 13.294 w where l and w are the length and width of a leaf respectively (Krishnakumar *et al.*, 1997)
No. of leaves	Counted twice in an year. The vine was tagged at the third leaf position and the number of leaves developed were recorded
Leaf phyllotaxy	Recorded for all the clones studied
Internodal length	Measured from third leaf axil to the adjacent leaf axil (cm)
Growth rate	The vines were tagged at the beginning of the work and the total growth in length was recorded twice in year (cm)
Root number	No. of roots emerging from third leaf axil was recorded
Root origin	Position from where the roots emerged was recorded

Floral and bean characters were not recorded due to the absence of flowering and fruiting during the observation period. All the observations were taken twice in a year.

3.2.1.2 Statistical analysis

Intraclonal variation was assessed by using the coefficient of variation. The interclonal variation among the clones derived from same pod, was studied using CRD.

To measure the overall performance of the plants from different pods and to find out the interclonal variation between plants from different pods Friedmann two way analysis of variance by ranks was adopted (Siegel, 1956).

3.2.2 Isozyme analysis

In order to observe whether the protein content in vanilla plants affected the isozyme analysis, the protein content of the twenty different accessions were estimated. Details are given below:

3.2.2.1 Protein estimation - Bradford Method

A. Reagents

1. Coomassie blue dye

Composition

Orthophosphoric acid	- 10 ml
Ethanol	- 5 ml
Coomassie blue G. 250	- 10 mg
Distilled water	- 85 ml

2. Bovine Serum Albumin (BSA) - 10 mg/10 ml of distilled water

The protein content of the plant samples was estimated using Bradford method (Sadasivam and Manickam, 1991). The solution was taken in 5 ml test tubes as given below.

Test tube No.	Blank	Standard	Sample	Duplicate
Distilled water	150 µl	140 µl	130 µl	130 µl
BSA	-	10 µl	-	-
Sample	-	-	20 µl	20 µl
Dye	2.8 ml	2.8 ml	2.8 ml	2.8 ml

The solution in each test tube was mixed gently and kept at room temperature for 5 minutes. They were then read at 595 nm using a spectrophotometer.

Esterase and peroxidase isozymes were used to evaluate the variability in vanilla. The isoforms of the enzyme were separated using native PAGE and stained with appropriate staining solution.

Acrylamide monomers ($CH = CHCONH_2$) were polymerised and cross-linked with N-N methylene bis acrylamide ($CH_2(NHCONH\text{-}CH_2)$ 2bis) to obtain the gel. Freshly prepared ammonium persulphate acted as chain initiator and N,N,N^1,N^1-tetra methyl ethylene diamine (TEMED) as catalyst.

Polyacrylamide gel was preferred because of its chemical inertness, high resolution, easiness in handling and preparation.

3.2.2.2 Preparation of reagents

A. Monomer solution (30% Acrylamide)

Composition

Acrylamide	: 58.4 g
Bis acrylamide	: 1.6 g
Distilled water to	: 200 ml

Solution was stored at 4°C in amber coloured bottles.

B. 4x Resolving gel buffer (1.5M Tris HCl, pH 8.8)

Composition

Tris base	: 36.8 g
Distilled water to	: 200 ml

The pH was adjusted to 8.8 with 1N HCl before making up the volume with distilled water and stored at 4°C.

C. 4x stacking gel buffer (0.5M Tris HCl, pH 6.8)

Composition

Tris base	: 6 g
Distilled water to	: 100 ml

The pH was adjusted to 6.8 with 1N HCl and stored at 4°C.

D. Tank buffer (0.025M Tris, pH 8.3)

Composition

Tris base	: 1.52 g
Glycine	: 7.2 g
Distilled water to	: 500 ml

The solution was stored at 4°C.

E. 10x treatment buffer/tracking dye (0.125M Tris HCl)

Composition

20X 2M Tris HCl, pH 6.8	: 1 ml
Glycerol	: 0.3 ml
Bromophenol blue (0.25M 1% solution)	: 300 µl
Distilled water to	: 2.0 ml

F. 10% Ammonium per sulphate(APS) (Chain initiator)

Composition

Ammonium persulphate	: 0.1 g
Distilled water	: 1 ml

This solution was prepared fresh, immediately before use.

G. TEMED - Original solution was used.

H. Extraction buffer (0.2M Tris HCl, pH 7.4)

Composition

Tris HCl	: 3.152 g
Distilled water to	: 100 ml

Tris HCl was dissolved in about 60 to 70 ml distilled water. The pH was adjusted using 10N NaOH to 7.4 and made up to 100 ml.

I. 0.5 M Phenol methane sulphonyl fluride (PMSF)

Composition

PMSF	: 25 mg
Isopropanol	: 1 ml

The solution was prepared by dissolving PMSF in isopropanol. It was prepared fresh.

J. 0.5 M Cysteine HCl

Composition

Cysteine HCl	: 50 mg
Distilled water to	: 1 ml

The solution was prepared fresh just before extraction.

K. Sucrose

L. Ascorbic acid

M. Staining solution for esterase

Composition

Sodium dihydrogen phosphate : 2.8 g

Disodium hydrogen phosphate : 0.015 g

Alpha-naphthyl acetate : 300 mg/5 ml Acetone

Fast blue RR salt : 0.1g

Distilled water to : 100 ml

The solution is prepared just before staining.

N. Staining solution for peroxidase

Composition

Sodium acetate : 54.2 ml

Acetic acid : 3.5 ml

Distilled water to : 100 ml

The solution was prepared just before staining. Sodium acetate and acetic acid was dissolved in water and to that 0.1g benzidine was added and dissolved. 1 ml of 3 percent hydrogen peroxide was added to the staining tray just before the gel was subjected to stain.

O. 7% Acetic acid (Destaining solution)

Composition

Acetic acid : 70 ml

Distilled water : 100 ml

The solution was prepared and stored at room temperature.

3.2.2.3 Preparation of gel

A. Casting of gel unit

The Hoefer Mighty Small™ gel system of Hoefer Pharmacia Biotech California was used. The glass plates were cleaned with tissue paper soaked in 95 percent ethanol. They were set apart by 0.75 mm thick spacer coated with sealing wax to avoid leak. The spacer and two glass plates were then assembled in the clamp. The clamp was tightened by aligning the glass plate spacer on the casting stand (Sadasivam and Manickam, 1991).

B. Resolving gel

Resolving gel was prepared by mixing the stock solution in the proportion given below.

Composition of 8 per cent resolving gel

Components	Quantity (ml)
Monomer	5.32
4x Resolving gel buffer	5.00
10% APS	0.20
TEMED	0.02
Distilled water	9.46
Total	20.00

C. Stacking gel

Composition of 4 per cent stacking gel

Components	Quantity (ml)
Monomer	1.332
4x stacking gel buffer	2.500
10% APS	0.100
TEMED	0.010
Distilled water	6.058
Total	10.000

The resolving gel was poured between the glass plates with the help of micropipette up to desired height (leaving 2 cm at the top). It was then carefully overlayed with distilled water. Polymerisation was allowed to take place for 1 hour. After complete polymerisation of resolving gel, distilled water was drained and stacking gel solution was carefully poured. The combs were placed properly and allowed to polymerise for 30 minutes.

After complete polymerisation (30 min), the combs were removed and the gel slab was carefully taken out from the casting unit, washed with the tank buffer and then transferred to electrophoretic apparatus. The upper and lower tanks were filled with pre-chilled tank buffer. The upper tank buffer was connected to cathode and lower to anode of the power pack (Hoefer Scientific Instruments, San Francisco).

3.2.2.4 Enzyme extraction

Tender and half mature leaves (I and III fully opened leaves) from the selected vanilla plants were used for extraction. Leaf samples were collected over ice early in the morning at 8.00 am. It was washed thoroughly and wiped with filter paper to remove moisture.

Four grams of fresh leaf sample were weighed and transferred to a prechilled mortar. To that 500 µl extraction buffer, 100 µl cysteine HCl, 100 µ PMSF, 10 µ ß mercaptoethanol, 350 mg sucrose and 440 mg ascorbic acid were added. It was then ground using an autoclaved prechilled pestle. A pinch of sterilised sand was added to make the grinding easier. The grinding was done at ice-cold condition. The ground sample was filled in 1.5 ml sterilized eppendorff tubes. The tubes were then centrifuged at 15,000 rpm for 15 minutes at 4°C in Bench top refrigerated centrifuge KUBOTA, Japan. The supernatant obtained was carefully transferred to a fresh sterilized 1.5 ml eppendorff tube and the amount of supernatant obtained was noted and used as enzyme source.

The crude sample was mixed with treatment buffer or tracking dye in the ratio of 1:9. From each sample, 180 µl was transferred into eppendorff tubes

containing 20 µl tracking dye and then 45 µl of sample - dye mixture was loaded in the wells. Five samples were loaded at a time. A duplicate gel was also loaded. A current of 10 mA per plate was maintained till the sample passed through the stacking gel. The current was increased to 15 mA per plate and was maintained through out the run till the tracking dye reached the anode.

3.2.2.5 Staining the gel

After the run, the gel was removed carefully and transferred to trays containing concerned stains. The staining was allowed in dark with intermittant shaking. The reaction was stopped with the destaining solution (90 minutes for estrase and 30 minutes for peroxidase) when the bands were sufficiently developed. The stained gels were preserved in distilled water for observation.

3.2.2.6 Documentation of the gel and analysis

Bands were visualised and was documented using documentation system (Alpha Imager-2000, Alpha Inotech, USA). Relative mobility (Rm) of the bands was noted as ratio of distance of band from origin to the distance of the dye front. Based on Rm values, the isoforms were named. Isoforms were scored as '0' for absence and '1' for presence on gel. The scores were then subjected to similarity index. A dendrogram was drawn depicting relationship of each plant based on SAHN clustering using NTSYS software. The bands were named after the enzymes and relative mobility (eg. EST 18 for esterase band with Rm 0.181 and PRX 10 for the peroxidase band with Rm 0.10). Based on banding pattern, a zymogram was also constructed.

3.3 MOLECULAR CHARACTERIZATION

3.3.1 DNA isolation

A modified CTAB extraction procedure reported by Doyle and Doyle (1987) was followed for the extraction of genomic DNA in vanilla. The quality of DNA is an important factor, which influence the PCR reactions.

Tender leaves were taken from the selected plants using sterile blades. The leaf samples were collected on ice, washed in tap water, rinsed and immediately used for extraction. Details are as follows:

A. Reagents

1. 4x Extraction buffer

Composition

Tris HCl	: 4.8 g
EDTA disodium salt	: 0.7 g
Sorbitol	: 25.6 g
Sterile milli Q water to	: 100 ml

Sorbitol, Tris HCl and EDTA were dissolved in about 80 ml sterile milli Q water and the pH was adjusted to 7.5 using concentrated HCl. The volume was then made up to 100 ml. 0.38g sodium metabisulphate (0.38 percent) was added prior to extraction.

2. Lysis buffer

Composition

1 M Tris HCl, pH 8	: 20.0 ml
0.25 M EDTA	: 20.0 ml
5 M NaCl	: 40.0 ml
Sterile Milli Q water	: 20.0 ml
Cetyl Trimethyl Ammonium Bromide (CTAB)	: 2.0 g

3. TE buffer

Composition

10 mM Tris HCl, pH 7.6	: 0.3028 g
1 mM EDTA	: 0.0930 g
Distilled water to	: 200 ml

4. Sarcosin (5%)

Composition

Sarcosin	: 5.0 g
Distilled water to	: 100 ml

All the reagents prepared above were autoclaved before use

5. Chilled isopropanol

6. Chloroform : isoamylalcohol mixture (24 : 1 v/v)

7. ß mercaptoethanol

7. Ethanol (100% and 70%)

B. Extraction procedure

0.5 g of the leaf sample collected was ground in a sterile mortar, with 3 ml of extraction buffer, 2.5 µl ß mercaptoethanol. The sodium meta bisulphate was added to that just before grinding. The homogenate was then poured into a centrifuge tube (50 ml) containing 7.5 ml lysis buffer and 1.25 ml (5%) sarcosin. The tube was heated for 15 minutes at 65°C and was occassionally mixed by inversion. Equal volume of chlorofom : isoamyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion and centrifuged at 10,000 rpm for 10 minutes at room temperature. The upper aqueous phase was pipetted out and saved in a 30 ml centrifuge tube. To the tube containing aqueous phase 2/3rd volume pre chilled isopropanol was added. The tube was kept at -20°C for ½ hour for precipitation. The contents were mixed gently by inversion until the DNA was precipitated. The DNA was pelleted by centrifuging at 10,000 rpm for 10 minutes at room temperature. The isopropanol was poured off, drained well and the pelleted DNA was washed with 70% alcohol and centrifuged at 10,000 rpm for 3 minutes. The pellet was air dried and resuspended in 200 µl TE buffer.

3.4 PURIFICATION OF DNA

The DNA isolated will also contain RNA and residual protein in it. To exclude the RNA and residual proteins, the sample was treated with RNase and proteinase K.

3.4.1 Preparation of RNase and proteinase

The Ribonuclease A from Geneii, Bangalore was used to prepare RNase. RNase was dissolved in 0.10M sodium acetate (pH 5.2) at the rate of 10 mg/ml. Solution was heated at 100°C for 15 minutes and allowed to cool to room temperature. The pH was adjusted to by adding 100 µl 1M Tris base (pH 7.4) and stored at -20°C.

The proteinase K at 20 mg ml^{-1} concentration (Bangalore Genei) was prepared in distilled water and stored at -20°C.

3.4.2 Incubation of DNA with RNase and proteinase

The extracted DNA suspended in TE buffer (100 µl) was treated with 20 µg 100 ml^{-1} of RNase solution and incubated at 37°C for 1 hour. The extracted DNA after RNase digestion was treated with 50 µg ml^{-1} proteinase K solution and incubated at 45°C for ½ hour.

After incubation, the sample was treated with equal volume (1:1) of phenol : chloroform-isoamyl alcohol mixture. It was then centrifuged at 10,000 rpm for 10 min at room temperature. The top layer was saved using a micropipette and transferred to a sterile eppendorff tube. To it equal volume of chloroform-isoamyl alcohol mixture was added and centrifuged at 10,000 rpm for 10 min at room temperature. The top layer obtained was again saved and centrifuged with equal volume of chloroform : isoamyl alcohol mixture. The final aqueous phase was saved in a sterile eppendorff tube and 2/3rd volume of chilled isopropanol was added, mixed gently and kept at -20°C for ½ hour until the DNA was precipitated. It was then centrifuged at 10,000 rpm for 10 min at room temperature. The isopropanol was poured off and the DNA pellet was washed first with 70 per cent alcohol and then with absolute alcohol. The DNA was then allowed to air dry, redissolved in 25 to 50 µl of TE buffer and stored at -20°C for further use.

3.5 ESTIMATION OF QUALITY AND QUANTITY OF DNA

The quality and quantity of isolated DNA were evaluated through electrophoresis and by spectrophotometric measurements.

3.5.1 Electrophoresis of DNA samples

The purity of the genomic DNA isolated from vanilla leaves was tested by carrying out agarose gel electrophoresis using the electrophoresis unit of Hoefer Pharmacia.

A. Reagents

1. Agarose

2. 50x TAE buffer

Composition

Tris base	: 242 g
0.5 M EDTA, pH 8	: 100 ml
Glacial acetic acid	: 57.1 ml

The contents were mixed well, autoclaved and stored at room temperature.

3. 6x Gel loading buffer

Composition

Bromophenol blue	: 0.25%
Xylene cyanol FF	: 0.25%
Glycerol in water	: 30%

The components were mixed well and stored at 4°C.

4. Ethidium bromide

Procedure

Gel buffer 1X TAE was prepared from the 50X TAE stock solution. Gel buffer 1X TAE was taken in a conical flask (100 ml for large gel and 30 ml for small). Agarose (1.0% for DNA samples and 1.4% for RAPD samples) was weighed, added to the flask, stirred and boiled in microwave oven till the agarose dissolved completely. 2 µl ethidium bromide was added into the flask, mixed well and it was allowed to cool to 40°C. The open end of the gel casting tray was sealed with cellotape and placed on a horizontal surface and the comb was placed properly on the tray. The dissolved agarose was poured gently into the tray. The gel was allowed to solidify for 30 minutes and then the comb was removed carefully. The gel was then placed in electrophoresis unit with the well side directed towards cathode. 1X TAE

buffer was added to cover the gel with 2 to 3 millimeter of buffer. 10µl of DNA sample (15 µl in case of RAPD products) was pipetted out onto a parafilm and mixed well with gel loading dye. The samples were then loaded carefully into the well using micropipette. Standard DNA molecular weight markers were loaded in one well and a negative control was also added in another well in case of electrophoresis after RAPD assay. The cathode and anode of the electrophoresis unit were then connected to the power supply and the gel was run at constant voltage (45 mA). The power supply was turned off when the loading dye moved to the required distance.

3.5.2 Gel documentation

The gel was taken from electrophoresis unit and viewed under UV light of 320 nm. The image of gel was monitored and stored in a gel documentation system (Alpha Imager-2000, Alpha Infotech, USA).

3.5.3 Quantification of DNA

After ensuring the quality of DNA in the sample by electrophoresis, 10 µl of it was diluted to 1.5 ml with sterile water and absorbance at 260 nm and 280 nm read against distilled water blank using UV visible spectrophotometer (Spectronic R Genesys 5). The purity of DNA was assessed from the ratio of OD value at 260 to OD value at 280. A ratio of 1.8 to 2.0 indicates pure DNA. The quantity of DNA in the pure sample was calculated using the formula.

OD260 = 1 is equivalent to 50 µg double stranded DNA/ml

Therefore, OD260 x 50 gives the quantity of DNA in µg/ml.

3.6 RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS

The procedure of Demeke *et al.* (1992) was modified and used for the amplification of genomic DNA isolated from vanilla samples. In Polymerase Chain Reaction, one cycle included:

(a) DNA denaturation at 92°C for 1 minute

(b) Annealing of the primer to the template DNA at 37.5°C for 1 minute

(c) Primer extension at 72°C for 2 minutes.

The PCR was programmed for 40 cycles to get proper amplification.

The reaction mixture (25 µl) consisted of:

1. 10x Assay buffer containing 15mM $MgCl_2$	- 2.5 µl
2. 1 mM dNTP mix	- 2.5 µl (200 µM)
3. Taq DNA polymerase	- 2.0 µl (0.6 units)
4. Primer	- 1.0 µl (5 p moles)
5. Template DNA	- 2.0 µl (50 ng)
6. Milli Q water	- 15.0 µl

A master mix without the template DNA and primer was prepared using the reaction mixture for the required number of reactions. From this master mix, 22.0 µl was pipetted into each PCR tube. 1.0 µl of primer and 2.0 µl of template DNA were added. The reaction mix was overlaid with 25 µl of sterile mineral oil. The PCR tubes were loaded in the Thermal Cycles (Eppendorff) and the programme was run using the following cycles. The programme was completed in 4 hours. The amplified products were electrophoresised on 1.5 per cent agarose gel using 1x TAE buffer. The gel was viewed under UV light in transilluminator and then documented using alpha imager.

3.6.1 Screening of random primers for RAPD

A total of 20 decamer primers (operon) under different series viz., OPE and OPF were screened for amplification of genomic DNA extracted from vanilla accessions, using the thermal cycles mentioned. From these, 10 primers that gave good amplification (3-10 bands) were selected and utilized for further characterization of 20 selected accessions.

3.6.2 Data analysis

The pattern of DNA amplification for the 10 primers was scored as 1 or 0 by the presence or absence of bands respectively and the data were analysed using NTSYS PC 2.0 software package. Similarity indices were computed as JACCARD's coefficient through 'Simqual' routine and clustering was done using Sequential Agglomerative Heirarchial Nested Clustering (SAHN) routine of the NTSYS package. A dendrogram was constructed for the 20 accessions based on the clustering using Unweighted Pair Group Method of Arithmatic Averages (UPGMA) by Sneath and Sokel (1973) using NTSYS package.

Results

4. RESULTS

The *in vitro* derived vanilla plants regenerated from same seed, seedlings from same pod and those from different pods were characterized using morphological, biochemical and molecular markers. The results obtained are described in this chapter.

4.1 MORPHOLOGICAL CHARACTERISATION

The morphological characters studied included leaf length, leaf width, leaf area, leaf phyllotaxy, leaf shape, internodal length, total growth, number of leaves, number of roots and origin of roots. The details were collected for accessions regenerated from same seed, seedling clones from same pod and those from different pods. The data collected are summarized in tables (Tables 1 to 5).

4.1.1 Intraclonal variation

Leaf length

All the accessions studied showed very low intraclonal variability in leaf length. The leaf length ranged between 12.43 cm and 13.50 cm in the accessions of clone a275 while it was only between 7.90 cm and 8.10 cm in the accessions of clone a777. The clone a82 possessed the highest average leaf length and clone a803 possessed the shortest average leaf length (Plate 2) (Table 1). The intraclonal variation in leaf length observed was very low in tissue culture derived vanilla plants from same seed.

Leaf width

Variation in leaf width ranged between 2.70 cm and 3.50 cm in the case of clone a370 while it was between 2.00 cm and 2.20 cm in the clone a803. Highest average leaf width was seen in clone a82 (4.00 cm) and the clone a803 possessed the smallest average leaf width (2.10 cm) (Plate 2).

Table 1. Intraclonal variation in morphological characters of TC derived plants in *Vanilla planifolia*

Pod No.	Clone No.	Accession No.	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Leaf phyllotaxy	Leaf shape	Internodal length (cm)
VP 47	a275	1.1.1	12.57	3.33		Alternate	Oblong-eliptic	4.20
		1.1.2	13.23	3.73		,,	,,	4.27
		1.1.3	13.50	3.60		,,	,,	4.00
		1.1.4	13.00	3.73		,,	,,	4.20
		1.1.5	12.43	4.13		,,	,,	3.90
Avg.			12.49	3.70	30.70	,,	,,	4.11
SD			0.45	0.29	3.76			0.16
CV			3.60	7.83	12.25			3.89
VP 67	a2	2.1.1	6.30	3.70		,,	,,	4.30
		2.1.2	6.53	3.50		,,	,,	4.60
		2.1.3	6.40	3.67		,,	,,	4.90
		2.1.4	6.60	3.60		,,	,,	4.20
		2.1.5	6.80	3.60		,,	,,	4.50
Avg.			6.52	3.61	7.83	,,	,,	4.50
SD			0.19	0.08	0.87			0.27
CV			2.91	2.21	11.11			6.00
VP 67	a13	2.6.1	12.60	3.50		,,	,,	3.60
		2.6.2	13.10	3.60		,,	,,	3.80
		2.6.3	12.90	3.60		,,	,,	4.10
		2.6.4	12.60	3.70		,,	,,	4.30
		2.6.5	12.50	3.80		,,	,,	3.40
Avg.			12.80	3.64	29.36	,,	,,	3.84
SD			0.24	0.11	0.98			0.36
CV			1.87	3.02	3.34			9.37
VPx4	a457	4.4.1	12.20	2.50		,,	,,	3.56
		4.4.2	11.96	2.10		,,	,,	3.86
		4.4.3	11.80	2.23		,,	,,	3.76
		4.4.4	11.80	2.40		,,	,,	4.03
		4.4.5	12.20	2.06		,,	,,	3.96
Avg.			11.99	2.258	8.26	,,	,,	3.83
SD			0.20	0.19	2.58			0.18
CV			1.67	8.41	31.25			4.69

Contd.

Table 1. continued.

Pod No.	Clone No.	Accession No.	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Leaf phyllotaxy	Leaf shape	Internodal length (cm)
VP70	a370	7.5.1	6.60	2.80		„	„	2.30
		7.5.2	6.70	2.90		„	„	2.30
		7.5.3	7.30	3.50		„	„	2.30
		7.5.4	6.80	2.70		„	„	2.20
		7.5.5	6.70	3.10		„	„	2.20
Avg.			6.82	3.00	3.55	„	„	2.26
SD			0.28	0.32	3.15			0.05
CV			4.10	10.40	88.85			2.21
VP 229	a555	9.5.1	11.20	3.30		Alternate	Oblong-eliptic.	6.30
		9.5.2	10.90	3.10		„	„	6.30
		9.5.3	10.90	3.00		„	„	5.90
		9.5.4	10.90	3.00		„	„	6.20
		9.5.5	10.70	3.10		„	„	6.30
Avg.			10.96	3.10	15.99	„	„	6.20
SD			0.19	0.12	2.00			0.17
CV			1.73	3.87	12.53			2.74
VP 228	a777	10.1.1	8.10	3.20		„	„	3.90
		10.1.2	8.00	3.10		„	„	4.20
		10.1.3	7.90	3.10		„	„	4.30
		10.1.4	8.00	3.00		„	„	4.10
		10.1.5	8.10	2.90		„	„	3.90
Avg.			8.02	3.06	5.51	„	„	4.080
SD			0.08	0.11	1.50			0.18
CV			0.99	3.59	27.25			4.41
VP 228	a803	10.3.1	3.60	2.10		„	„	3.70
		10.3.2	3.40	2.20		„	„	3.80
		10.3.3	3.70	2.00		„	„	3.90
		10.3.4	3.60	2.10		„	„	4.00
		10.3.5	3.30	2.20		„	„	3.80
Avg.			3.52	2.12	22.18	„	„	3.84
SD			0.16	0.08	0.61			0.11
CV			4.54	3.77	2.75			2.84

Contd.

Table 1. continued.

Pod No.	Clone No.	Accession No.	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Leaf phyllotaxy	Leaf shape	Internodal length (cm)
VP 46	a82	11.5.1	15.10	3.90		,,	,,	6.70
		11.5.2	14.90	4.10		,,	,,	6.90
		11.5.3	15.10	4.00		,,	,,	6.90
		11.5.4	14.30	3.90		,,	,,	7.10
		11.5.5	15.10	4.10		,,	,,	7.00
Avg.			14.90	4.00	1.23	,,	,,	6.920
SD			0.35	0.10	2.12			0.15
CV			2.35	2.54	5.14			2.17
VP153	a349	22.1.1	15.10	3.10		,,	,,	4.80
		22.1.2	15.00	3.20		,,	,,	5.10
		22.1.3	14.80	3.30		,,	,,	5.10
		22.1.4	14.50	3.30		,,	,,	4.80
		22.1.5	14.20	3.40		,,	,,	4.70
Avg.			14.72	3.26	30.79	,,	,,	4.90
SD			0.37	0.11	0.60			0.19
CV			2.51	3.37	1.95			3.88

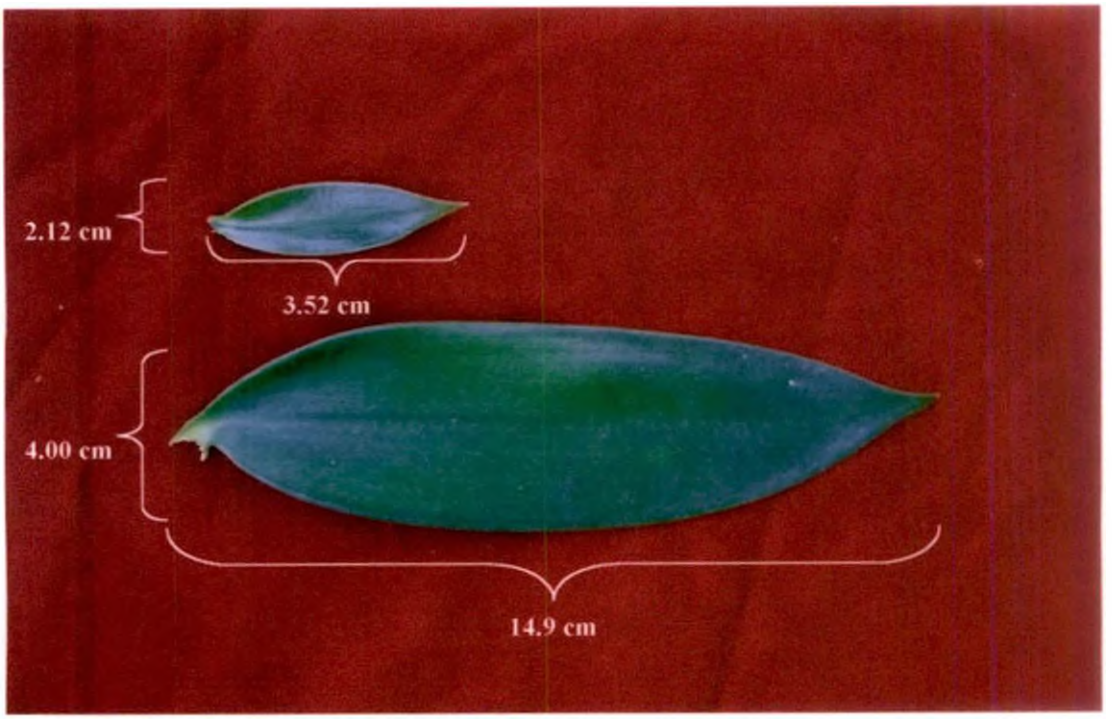

Plate 2. Variation in leaf size observed in *in vitro* derived vanilla clones

1 **2**

Plate 3. Leaf phyllotaxy in *in vitro* derived vanilla plants

1) Alternate with zigzag growth
2) Alternate with normal growth

Leaf area

The leaf area ranged from 3.54 cm^2 to 41.23 cm^2. There was a considerable variation in the accessions derived from the clones a370, a457 and a777 with coefficient of variations 88.85, 31.25 and 27.25 per cent respectively. The coefficient of variation was comparitively low in the other clones derived from same seed (Table 1).

Leaf shape and leaf phyllotaxy

Leaf shape in vanilla was generally oblong-elliptic in all the clones (Table 1). However the ratio between length and breadth varied in different clones (Plate 2).

Alternate leaf phyllotaxy was observed in all the clones(Table 1). Certain clones a275 (VP47), a362 (VP70), a803 (VP228), a128 (VP90), a79 (VP46), a307 (VP156), a305 (VP156), a459 (VPX-4), exhibited a zig-zag pattern of growth (Plate 3).

Internodal length

Internodal length ranged between 2.20 cm and 4.30 cm in the clones studied for intraclonal variation. Intraclonal variation recorded was not very high. The highest variation was observed in the clone a13 in which the internodal length varied from 3.40 cm to 4.30 cm. The clone a82 recorded the longest internodal length (6.90 cm) and a370 possessed the shortest internode (2.60 cm) (Plate 4) (Table 1). A general comparison of different clones varying in shoot characters is presented in Plate 5.

Total growth

The total annual growth among the different clones varied from 97 cm to 695 cm. The clones derived from the clone a204 showed very low variation with the total growth ranging between 647 cm to 695 cm whereas the accessions derived from the clone a32 showed total growth variation from 82 cm to 123 cm (Table 2).

Plate 4. Variation in internodal length observed in *in vitro* derived vanilla clones

Plate 5. Morphological variability in *in vitro* seedling derived clones of vanilla

Number of leaves

The total number of leaves produced in an year ranged between 21 to 87. The variation observed within the clone ranged between 4 to 12. Clone a32 showed the total number of leaves ranging from 21 to 25 whereas it was from 75 to 87 in clone a204.

Number of roots and root origin

In all the clones studied, roots were born singly opposite to the leaves. No variation was observed in this regard (Table 2) (Plate 6).

4.1.2 Interclonal variation within the pod

Interclonal variation was observed in tissue culture derived plants derived from different seeds of the same pod. The data recorded are presented in Tables 3 and 4.

Leaf length

Leaf length recorded for different clones derived from the same pod ranged between 3.52 cm and 17.32 cm. The variation observed was found statistically significant. Leaf length ranged between 7.30 cm and 12.66 cm in the clones from the pod VP229 while the variation observed was only between 11.74 cm and 13.58 cm in the ones derived from the pod VP91. Variability was maximum in the clones derived from pod VP229 with a standard deviation of 3.38. The accessions derived from the pods VP67, VPX4, VP91, VPX1, VP229(2), VP46, VP47, VPX3 and VP229(1) showed significant variation (Table 3).

Leaf width

Clones derived from the pod VPX4 showed highest variation, with leaf width ranging from 2.25 cm to 4.79 cm. The clones obtained from the pod VP47 showed least variation i.e., between 3.70 cm and 4.50 cm (Table 3).

Plate 6. Root origin and number of roots originated in *in vitro* derived vanilla plants

Table 3. Interclonal variation in morphological characters of tissue culture plants derived from same pod.

Pod	Clone No.	Leaf length (cm)	Leaf width (cm)	Leaf area (cm)	Internodal length (cm)
VP 47	a275	12.946	2.704	30.701	4.114
	a331	16.426	3.948	45.693	3.546
	a332	12.078	3.920	30.642	4.040
	a94	15.644	4.544	50.976	9.446
	a106	11.988	3.906	30.152	4.246
Avg		13.82	4.00	37.63	5.08
CD		2.49	NS	NS	0.90
VP 167	a2	6.526	3.614	7.830	4.500
	a46	10.900	3.800	25.070	6.580
	a47	11.980	4.460	37.490	7.860
	a58	14.320	4.260	42.731	6.460
	a32	8.260	3.620	13.764	4.040
	a13	12.800	3.640	29.357	3.840
Avg		10.80	3.90	26.04	5.57
CD		1.22	0.36	8.68	0.93
VPx3	a436	8.420	2.244	12.066	5.200
	a425	12.800	3.664	29.676	4.292
	a427	12.744	3.618	37.602	3.230
	a430	13.826	4.062	38.431	3.838
	a428	10.782	3.636	22.491	6.132
	a434	13.230	3.216	25.172	4.678
Avg		11.97	3.41	27.57	4.56
CD		2.63	0.68	10.90	0.61
VPx4	a439	10.590	1.918	10.482	4.810
	a451	12.804	4.790	44.659	4.502
	a454	15.080	4.424	44.477	4.290
	a457	11.992	2.258	8.257	3.834
	a446	12.938	2.646	16.609	2.170
	a453	13.204	4.326	39.841	3.330
Avg		12.768	3.39	26.38	3.82
CD		1.02	0.44	8.15	0.17
VP91	a136	13.580	5.220	52.995	6.500
	a159	12.580	5.320	50.948	4.980
	a137	12.360	4.640	41.166	5.960
	a139	11.740	4.220	33.489	6.440
	a204	12.100	4.000	31.780	6.940
Avg		12.472	4.68	42.07	6.16
CD		NS	0.46	7.81	0.71

Contd.

Table 3. continued

Pod	Clone No.	Leaf length (cm)	Leaf width (cm)	Leaf area (cm)	Internodal length (cm)
VP229(1)	a962	9.880	3.760	21.014	5.460
	a866	7.300	2.480	5.022	2.780
	a861	12.660	4.480	40.051	6.180
	a712	17.320	4.520	56.315	8.640
	a710	10.240	3.180	16.059	8.220
Avg		11.48	3.68	27.70	6.25
CD		1.58	0.54	10.68	0.51
VP70	a319	11.040	3.200	17.566	2.840
	a362	12.020	3.460	27.816	6.520
	a410	14.480	3.500	33.167	3.960
	a396	6.640	2.740	4.037	3.140
	a370	6.820	3.000	3.545	2.260
Avg		10.20	3.18	17.22	3.744
CD		1.98	NS	26.07	1.0
VPx1	a468	8.598	2.900	8.287	3.360
	a481	9.360	2.560	6.710	3.860
	a520	11.280	3.260	19.174	5.440
	a479	9.840	2.820	8.463	3.500
	a489	12.300	3.620	27.403	5.440
	a448	13.940	3.620	32.940	5.360
Avg		10.80	3.13	17.16	4.49
CD		1.26	0.29	6.39	0.49
VP229(2)	a580	12.70	3.70	29.817	3.100
	a859	10.74	3.40	19.212	3.620
	a720	10.44	3.24	16.072	2.700
	a557	10.78	3.38	19.081	3.120
	a555	10.96	3.10	15.966	6.200
	a544	15.80	5.00	57.565	3.420
Avg		11.90	3.63	26.28	3.69
CD		1.46	0.41	9.49	0.29
VP228	a777	8.020	3.060	5.509	4.080
	a820	4.920	2.160	16.921	2.000
	a803	3.520	2.120	22.179	3.840
	a854	11.100	3.380	20.574	3.800
	a887	8.420	2.520	5.596	4.340
Avg		7.196	2.64	14.15	3.612
CD		1.37	0.27	24.90	0.78
VP40	a81	8.700	3.240	10.198	2.920
	a83	11.040	3.120	16.502	3.240
	a84	11.340	4.060	30.011	4.340
	a79	11.300	3.640	24.293	2.800
	a82	14.900	4.000	41.232	6.920
Avg.		11.456	3.612	24.45	4.044
CD		1.46	0.76	1.41	1.03

Leaf area

The pod VP228 showed a variation between 5.51 cm^2 and 22.18 cm^2 in its clones whereas those from the pod VP229(2) showed a variation ranging from 15.97 cm^2 to 57.57 cm^2. Interclonal variation between the different clones derived from the same pod was highly significant in all the clones. The clones from the pod VP47 showed no significant variation in their leaf area (Table 3).

Internodal length

Considerable variation for the internodal length was observed in the clones derived from the same pod. Highest variation was shown by the clones obtained from pod VP47, with internodal length ranging from 3.54 cm to 9.44 cm and the lowest observed was between 4.98 cm and 6.90 cm in the clones from VP91. Interclonal variation with respect to internodal length was significant (Table 3).

Total growth

The clones obtained from the pod VPX4 showed the higher variation between 102.2 cm and 586.0 cm while those from the pod VP70 showed a lower variation from 365 cm to 569 cm. The clones obtained from the same pod varied significantly in total growth, except for those accessions derived from pod VP47 and VP70, where there was no significant difference statistically (Table 4).

Number of leaves

The interclonal variation in number of leaves was found highly significant in all the clones from the same pod except for those from pod VP47. The clones from pod VPX4 showed a maximum variation in number of leaves, ranging from 28 to 80 leaves while those from pod VPX1 showed minimum variation from 39 to 54 leaves (Table 4).

4.1.3 Variation between the plants from different pods in vanilla

The interclonal variation recorded between the different pods are described in Tables 5 and 6.

Table 4. Interclonal variation in growth rate and root characters of tissue culture plants derived from same pod.

Pod No.	Clone No.	Total growth	No. of leaves	No. of roots	Root origin
VP47	a275	423.4	58	Single	Opposite to leaf
	a331	579.4	65	,,	,,
	a332	489.0	58	,,	,,
	a94	664.6	74	,,	,,
	a106	448.8	57	,,	,,
Avg.		521.1	62	,,	,,
CD		NS	NS		
VP67	a2	320.4	38	,,	,,
	a46	553.8	79	,,	,,
	a47	603.6	70	,,	,,
	a58	476.6	62	,,	,,
	a32	110.4	24	,,	,,
	a13	428.0	50	,,	,,
Avg.		498.5	54	,,	,,
CD		52.91	4.85		
VPx3	a436	319.2	55	,,	,,
	a425	454.0	48	,,	,,
	a427	284.0	40	,,	,,
	a430	410.4	55	,,	,,
	a428	625.0	61	,,	,,
	a434	498.0	47	,,	,,
Avg.		431.7	51	,,	,,
CD		62.11	6.20		
VPx4	a439	586.0	80	,,	,,
	a451	103.2	29	,,	,,
	a454	285.4	47	,,	,,
	a457	399.8	72	,,	,,
	a446	102.2	28	,,	,,
	a453	286.0	61	,,	,,
Avg.		293.7	53	,,	,,
CD		40.08	5.90		
VP91	a136	703.8	91	,,	,,
	a159	443.9	69	,,	,,
	a137	469.2	49	,,	,,
	a139	579.0	68	,,	,,
	a204	678.6	81	,,	,,
Avg.		574.9	72	,,	,,
CD		36.98	4.15		

Contd.

Table 4. Continued

Pod No.	Clone No.	Total growth	No. of leaves	No. of roots	Root origin
VP299	a862	268.6	45	Single	Opposite to leaf
	a866	219.6	35	,,	,,
	a861	391.2	43	,,	,,
	a712	518.2	58	,,	,,
	a710	441.0	51	,,	,,
Avg		367.7	46	,,	,,
CD		54.57	5.42		
VP70	a369	443.0	54	,,	,,
	a362	387.4	51	,,	,,
	a410	365.0	38	,,	,,
	a396	569.0	57	,,	,,
	a370	396.0	37	,,	,,
Avg.		432.0	47	,,	,,
CD		NS	6.83		
VPx1	a468	172.0	41	,,	,,
	a481	189.0	43	,,	,,
	a520	429.0	54	,,	,,
	a479	376.0	41	,,	,,
	a489	297.8	39	,,	,,
	a448	453.6	49	,,	,,
Avg.		319.5	44.5	,,	,,
CD		50.23	6.59		
VP229	a580	340.7	53	,,	,,
	a859	213.9	29	,,	,,
	a720	388.0	54	,,	,,
	a557	605.0	73	,,	,,
	a555	600.0	64	,,	,,
	a544	410.4	37	,,	,,
Avg.		426.2	52	,,	,,
CD		55.13	5.10		
VP228	a777	166.0	36	,,	,,
	a820	278.2	21	,,	,,
	a803	403.7	60	,,	,,
	a854	310.0	52	,,	,,
	a887	201.0	57	,,	,,
Avg.		271.7	45	,,	,,
CD		54.80	7.90		
VP46	a81	162.0	35	,,	,,
	a83	325.0	47	,,	,,
	a84	267.0	42	,,	,,
	a79	345.6	41	,,	,,
	a82	569.8	55	,,	,,
Avg.		333.8	44	,,	,,
CD		41.57	11.69		

Leaf length

The pod VP153 showed the maximum average leaf length of 14.72 cm and the pod VP228 showed the minimum average leaf length of 7.19 cm. The standard deviation observed interclonally between different pods in leaf length was 1.93. The coefficient of variation was found to be 16.38 per cent (Table 5).

Leaf width

Leaf width ranged between 2.24 cm and 4.74 cm in the pods VP90 and VP159 respectively. The standard deviation was found to be 0.61 and the coefficient of variation was 18.20 per cent.

Leaf area

Leaf area varied from 3.231 cm^2 to 50.069 cm^2 in the plants from different pods studied. The plants from pod VP90 had an average leaf area of 3.23 cm^2 while the plants from pod VP159 had an average leaf area of 50.07 cm^2. High variability was observed for leaf area in plants derived from different pods with standard deviation 10.38 and a coefficient of variation of 41.04 per cent (Table 5).

Total growth

The vanilla plants derived from different pods varied significantly with respect to total growth. Maximum, average total growth was observed in the plants derived from pod VP90 with average growth of 643.00 cm while minimum growth was recorded in the plants derived from pod VP52 with average growth of 249.10 cm. The standard deviation was 119.70 and the coefficient of variation was 27.50 per cent (Table 6).

Number of leaves

Pod VP159 showed a highest leaf number of 76 whereas pod VP52 showed the least number of leaves, 30 (Table 6).

Table 5. Interclonal variation in morphological characters of tissue culture derived plants from different pods

Pod	Mean leaf length (cm)	Mean leaf width (cm)	Leaf area (cm^2)	Internodal length (cm)
VP47	13.82	4.00	37.63	5.08
VP67	10.80	3.90	26.04	5.57
VPx3	11.97	3.41	27.57	4.56
VPx4	12.77	3.39	26.38	3.82
VP91	12.47	4.68	42.07	6.16
VP229	11.48	3.68	27.70	6.25
VP70	10.20	3.18	17.22	3.74
VPx1	10.80	3.13	17.16	4.49
VP228	7.19	2.64	14.15	3.61
VP46	11.46	3.61	24.45	4.04
VP156	11.86	3.26	22.48	7.11
VP90	8.66	2.24	3.23	4.06
VP155	13.08	3.76	31.89	7.58
VP230	13.63	3.83	34.68	6.04
VP159	14.60	4.74	50.59	10.88
VP153	14.72	3.26	30.70	4.90
VP52	10.80	3.24	17.29	3.14
Avg.	11.78	3.35	26.51	5.35
SD	1.93	0.61	10.88	1.63
CV	16.38	18.20	41.04	30.46

Table 6. Interclonal variation in growth rate of tissue culture plants derived from different pods.

Pod	Avg. Total growth (cm)	Avg. No. of leaves
VP47	521.1	62
VP67	498.5	54
VPx3	431.7	51
VPx4	293.7	53
VP91	574.9	72
VP229	367.7	46
VP70	432.0	47
VPx1	319.0	44
VP228	271.7	45
VP46	333.8	44
VP156	446.2	64
VP90	643.0	73
VP155	392.1	48
VP230	418.0	59
VP159	611.3	76
VP153	598.4	72
VP52	249.1	30
Avg	435.4	55
SD	119.7	12.55
CV	27.5	22.69

The Friedmann test for vegetative characters of clones derived from 17 different pods indicated lot of variability among the clones derived from different pods. According to the test values, the pods were ranked based on variability as shown in Table 7. Higher the ranking scores higher the vegetative characters. The ranking scores ranged from 2.17 to 16.67 for the clones derived from pods VP228 and VP159 respectively.

Floral and bean characters were not recorded due to the absence of flowering and fruiting during the period of observation.

4.2 ISOZYME ANALYSIS

4.2.1 Protein estimation

Bradford's method of quantitative estimation of protein was carried out. The quantity of the protein varied from 0.063 mg ml^{-1} to 0.552 mg ml^{-1} extract (Table 8).

4.2.2 Esterase activity

4.2.2.1 Standardization of leaf sampling

In both the samples, viz., first and third fully opened leaf from the tip, 10 bands were observed for esterase with Rm ranging from 0.181 to 0.752. The banding pattern also was same for both the samples. The third leaf was selected as ideal for the analysis due to the clear and thick bands (Plate 7).

4.2.2.2 Esterase variation in in vitro derived vanilla plants

The banding pattern is depicted in Fig.1. Altogether 10 isoforms were observed for esterase enzyme in vanilla and were named as EST 18, EST 20, EST 24, EST 30, EST 38, EST 43, EST 50, EST 60, EST 70 and EST 75. Out of these EST 50 and EST 60 were monomorphic except for a370 (EST 60), a 58 (EST 50) and a434 (EST 50) i.e., were present in 95 percent and 90 percent respectively in the accessions studied. The band EST 24 and EST 38 were common for the accessions a439, a448, a453, a454 and a446. The accessions a425, a439, a448, a453, a454, a446, a481 and

Table 7. Friedmann test for studying interclonal variation between different pods

Pods	Ranking order
VP47	13.33
VP67	10.00
VPx3	9.00
VPx4	7.50
VP91	14.08
VP229	9.17
VP70	5.00
VPx1	4.08
VP228	2.17
VP46	6.08
VP156	10.08
VP90	7.17
VP155	11.33
VP230	12.00
VP159	16.67
VP153	12.33
VP52	3.00

Table 8. Protein content of different accessions estimated by Bradfords method

Sl. No.	Accessions	Protein content (mg/ml)
1	a425	0.544
2	a136	0.165
3	a439	0.173
4	a159	0.150
5	a520	0.410
6	a448	0.390
7	a46	0.063
8	a453	0.210
9	a454	0.190
10	a446	0.227
11	a47	0.105
12	a481	0.454
13	a427	0.230
14	a58	0.255
15	a82	0.240
16	a428	0.180
17	a396	0.171
18	a370	0.194
19	a555	0.133
20	a468	0.552

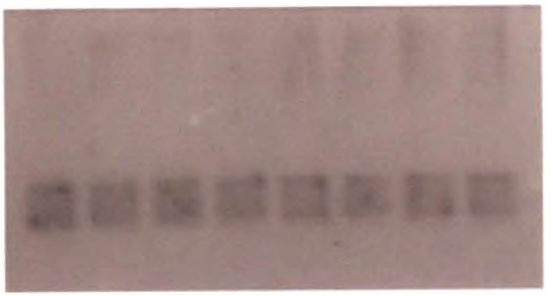

Plate 7. Standardization of leaf sampling for esterase analysis

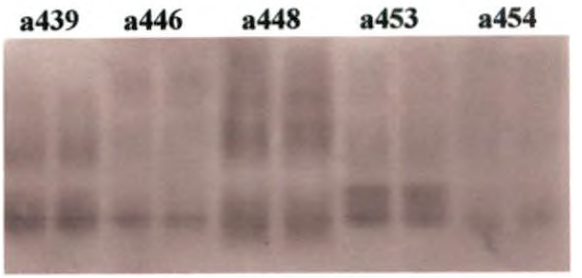

Plate 8. Esterase isozyme banding pattern in *in vitro* derived vanilla plants-I

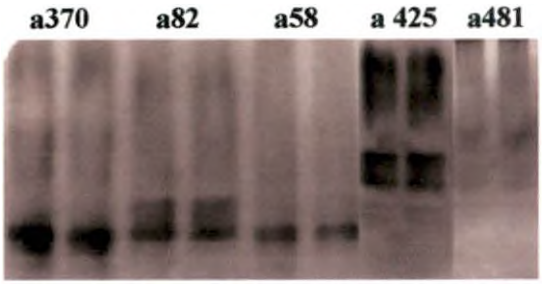

Plate 9. Esterase isozyme banding pattern in *in vitro* derived vanilla plants-II

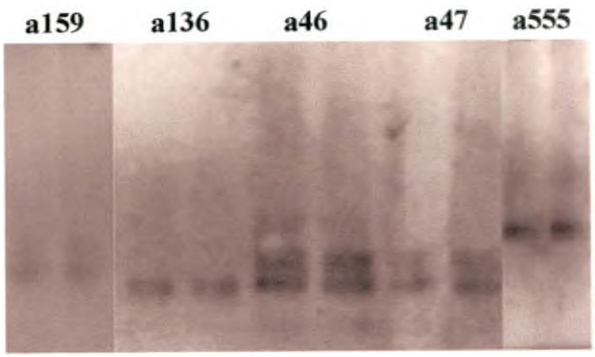

Plate 10. Esterase isozyme banding pattern in *in vitro* derived vanilla plants-III

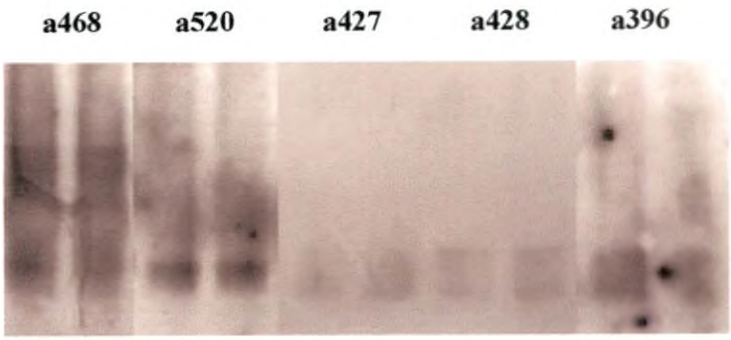

Plate 11. Esterase isozyme banding pattern in *in vitro* derived vanilla plants-IV

a468 had the band EST 30 in common and the band EST 20 was present in the accessions a425, a396 and a370. The accessions a82 and a370 had the band EST 18 in common and accessions a425 and a481 had band EST 75 in common. The band EST 43 was specific for the accession a370 and the band EST 70 was specific for a425 (Plates 8, 9, 10 and 11).

Thus EST 50 and EST 60 were the unique esterase isoforms of vanilla.

4.2.2.3 Cluster analysis

Dendrogram plotted from the data obtained indicated distinct variation among the vanilla plants. The 20 different accessions formed nine clusters. Cluster I was a solitary one formed by accession a82 and cluster II comprised of the accessions a520, a427, a428, a159, a47, a46, a13 and a555. Cluster III, IV and V were solitary consisting of only one accession under each, namely, a396, a58 and a370. The next one, cluster VI comprised of the accessions a439, a446, a448, a453 and a454. The next three clusters VII, VIII and IX were solitary formed by the accessions a481, a468 and a425 respectively. The accessions a82, a396, a58, a370, a468, a481 and a425 formed distinct clusters and were found highly dissimilar. The accession a370 of pod VP70 was highly variable with a coefficient of variation of 60 per cent whereas a481 and a468 derived from pod VP1 showed 25.4 per cent coefficient of variation. There were significant variation among the different accessions. Also the variation between the pods were found significant (Fig.2).

4.2.3 Peroxidase activity

4.2.3.1 Standardisation of leaf sampling

The leaf samples taken was as that of esterase. Twelve bands were observed. The Rm values ranged from 0.100 to 0.705. The banding patterns were same for first and third fully opened leaf. The bands were deep and thicker in case of third leaf sample. Hence third leaf was selected as ideal one, for the analysis (Plate 12).

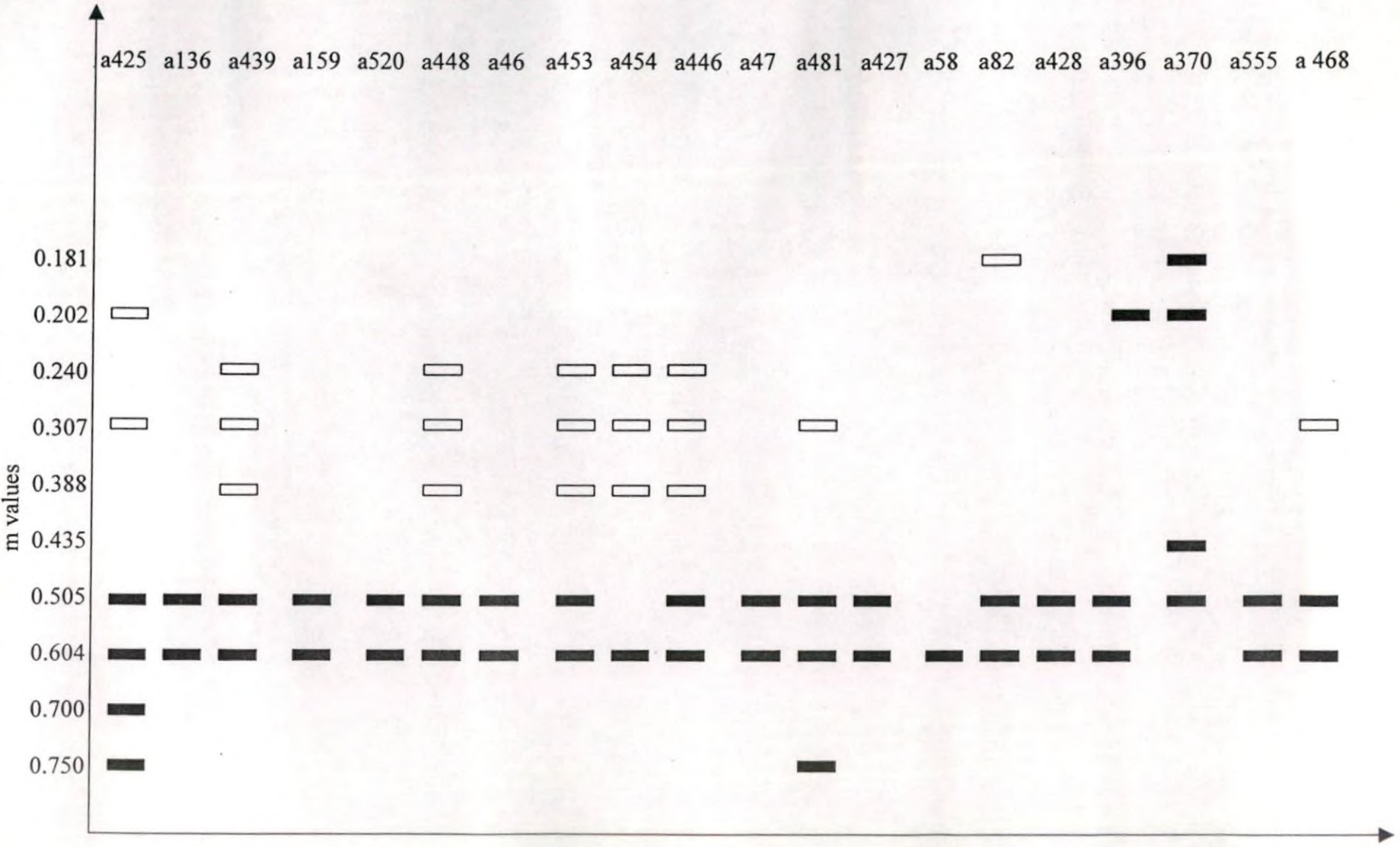

Fig. 1. Esterase zymogram of the 20 accessions

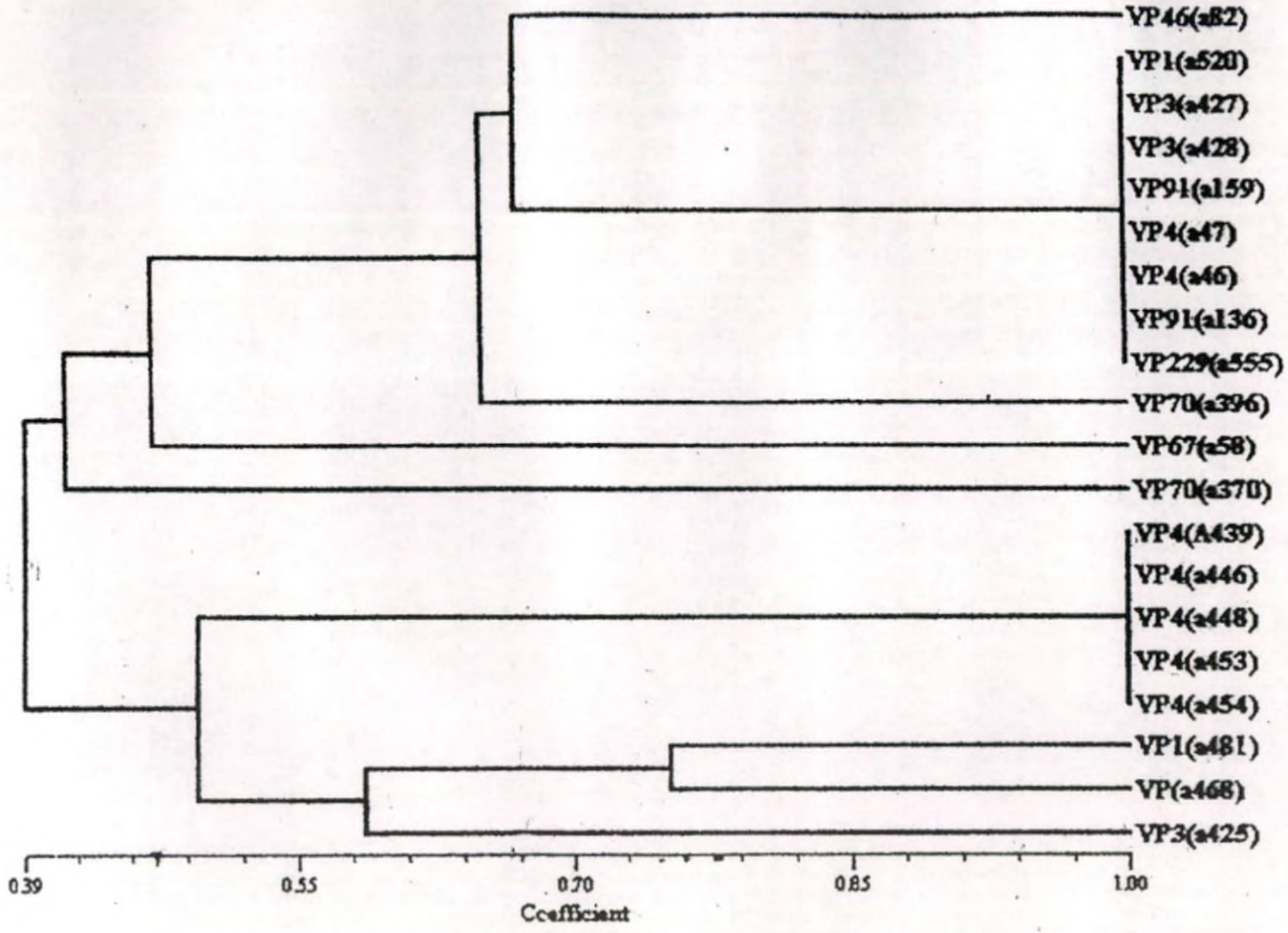

Fig. 2. Dendrogram showing the different clusters formed by the 20 different clones of vanilla for esterase isozyme

4.2.3.2 Peroxidase variation in in vitro derived vanilla plants

The zymogram of peroxidase activity is furnished in Fig.3. Out of the 12 bands observed in peroxidase, three bands (PRX 56, PRX 50 and PRX 60) were monomorphic or common for most of the accessions. The accession which lacked the common band PRX 56 was a136, hence the percentage of occurrence of the band was 95 percent. The bands PRX 50 and PRX 60 had 70 and 75 percent occurrence. The band PRX 65 was common in the accessions a439, a159, a446, a481, a427, a58, a82, a396, a555 and a468. The accessions a439, a159, a448, a453, a454, a446, a481, a427 and a428 had the band PRX 47 in common and the band PRX 70 was present in a136, a481, a427, a58, a82, a396 and a468. The band PRX 50 was present in the accessions a425, a481, a58, a82, a396, a370 and a555. The band PRX 50 was common for a425, a439, o446 and 0.481. Four accessions a159, a47, a481 and a58 two bands PRX 17 and PRX 24 in common. The band PRX 40 was specific for two accessions, a425 and a481. The band PRX 10 was unique in accession a159 (Plates 13,14,15 and 16).

4.2.3.3 Cluster analysis

Dendrogram plotted from the data showed that the 20 clones formed 15 groups. High variability was observed for the peroxidase banding pattern among the clones studied. The clone a425 showed highest dissimilarity of 80 per cent, while some other clones like a454 and a468; a439 and a446; a448 and a452 were almost uniform. Distinct banding patterns were observed for the other clones studied indicating the distinct nature or heterogeneity of the clones studied (Fig.4).

4.3 DNA MARKERS

4.3.1 Genomic DNA isolation in vanilla

Genomic DNA was isolated from 20 tissue culture regenerants following the modified Doyle and Doyle method of DNA isolation. The protocol was found effective and the high interference of RNA was nullified by RNase treatment. The quality and quantity of the isolated DNA are presented in Plate 17 and Table 9. The electrophoretic profile showed clear narrow bands. The ratio of absorbance at 260nm and at 280nm was 1.811 and the quantity of DNA estimated was around 48mg g^{-1}.

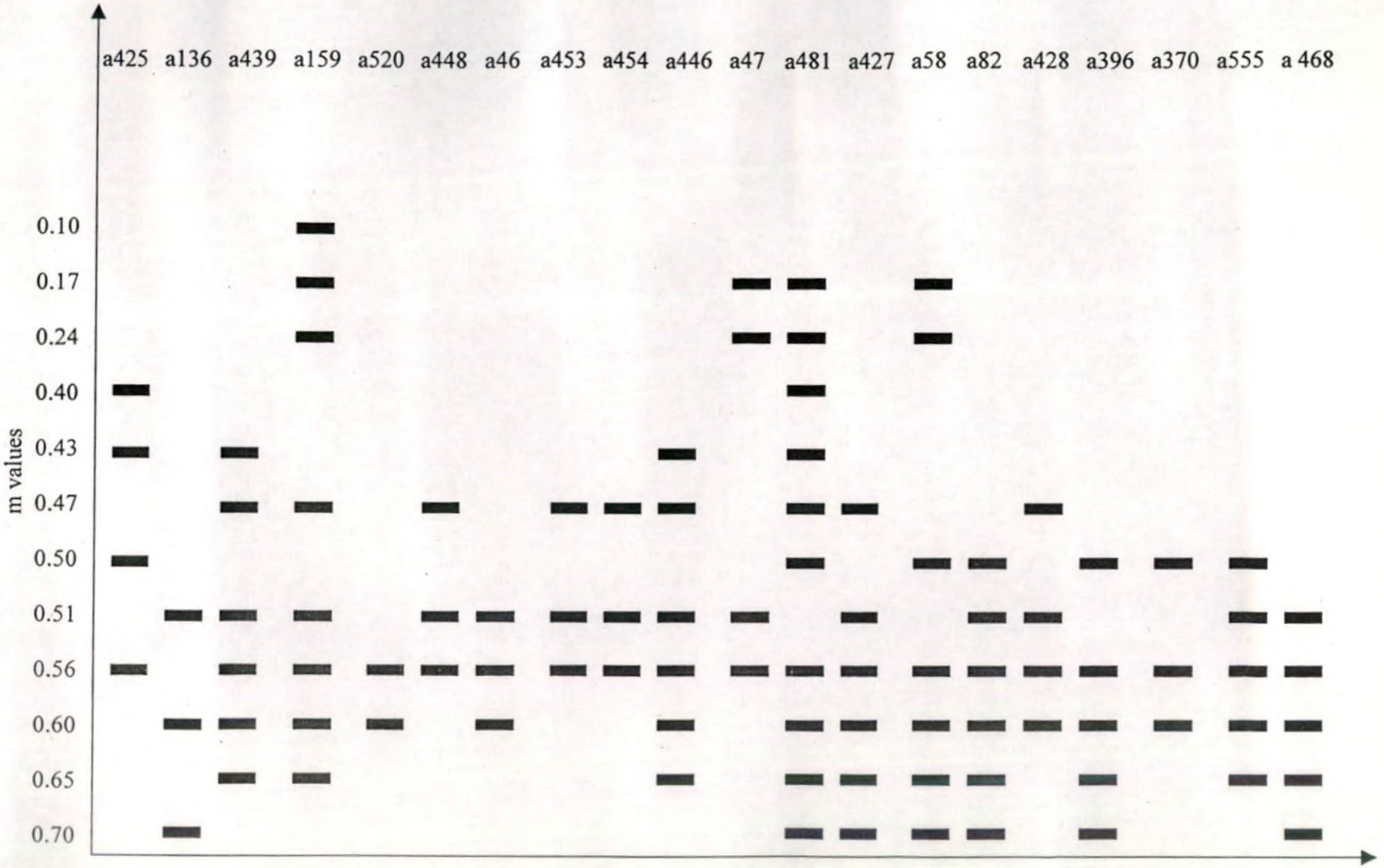

Fig. 3. Peroxidase zymogram of the 20 accessions

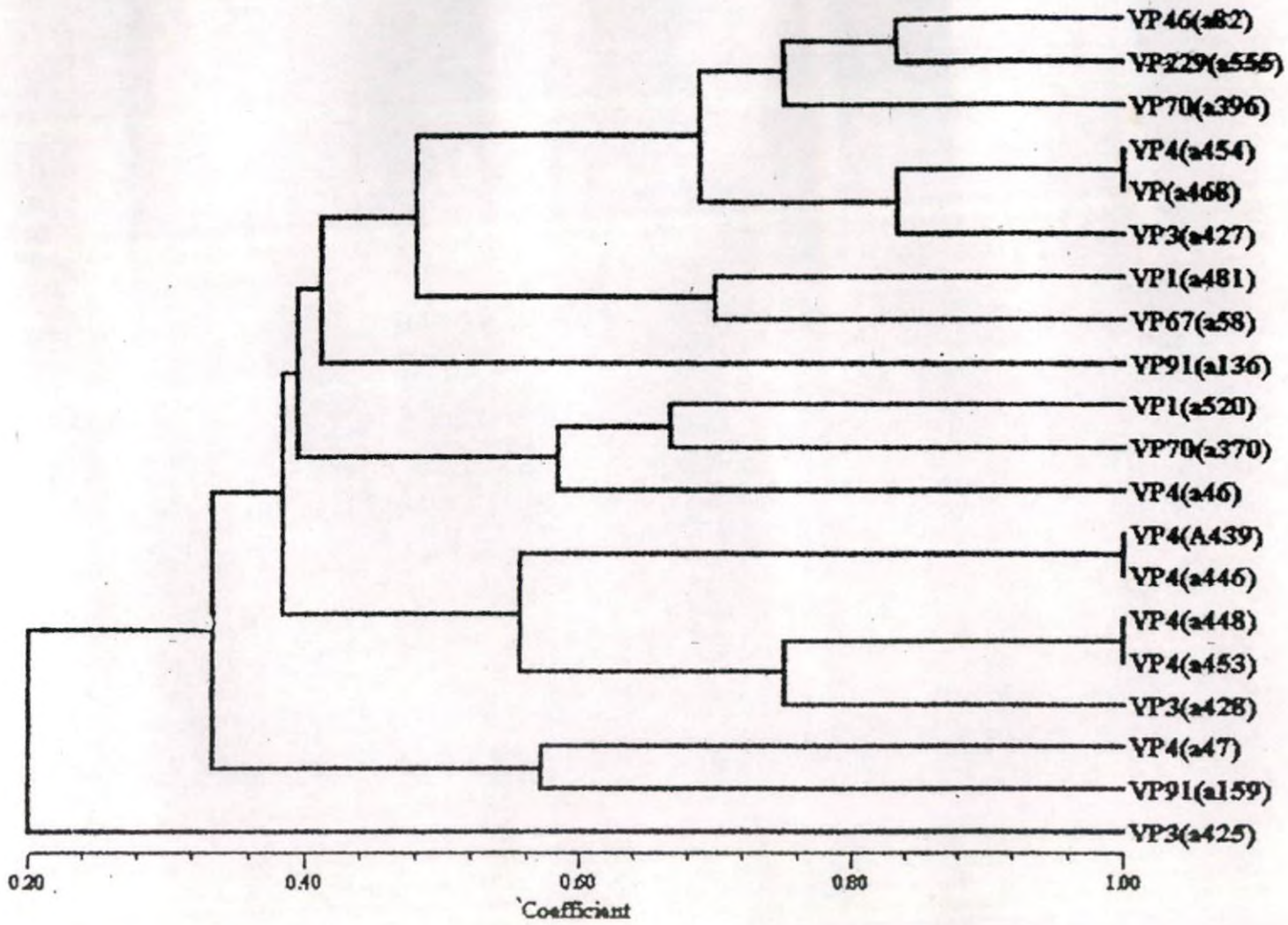

Fig. 4. Dendrogram showing the different clusters formed by the 20 different clones of vanilla for peroxidase isozyme

Plate 12. Standardization of leaf sampling for peroxidase analysis

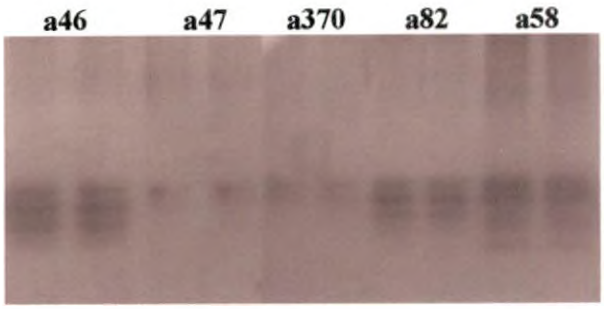

Plate 13. Peroxidase isozyme banding pattern in *in vitro* derived vanilla plants-I

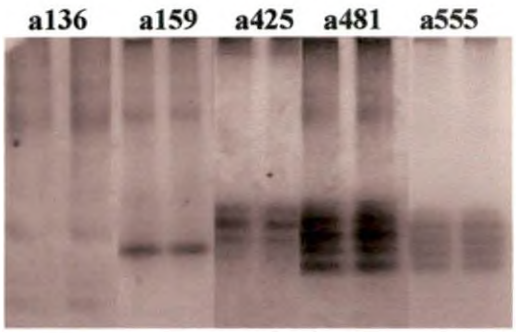

Plate 14. Peroxidase isozyme banding pattern in *in vitro* derived vanilla plants-II

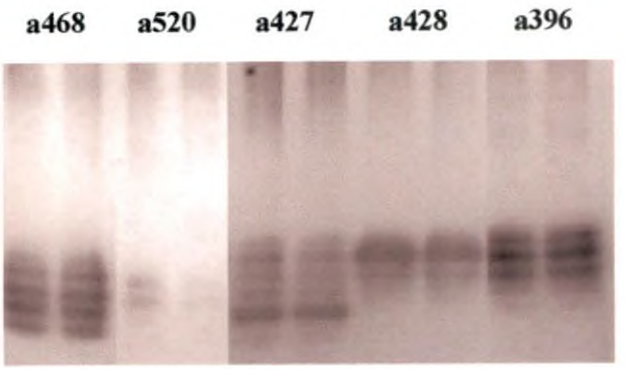

Plate 15. Peroxidase isozyme banding pattern in *in vitro* derived vanilla plants-III

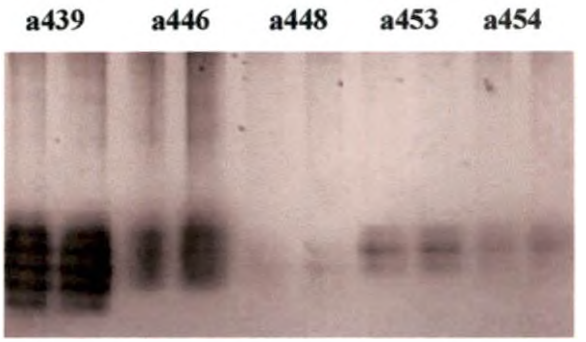

Plate 16. Peroxidase isozyme banding pattern in *in vitro* derived vanilla plants-IV

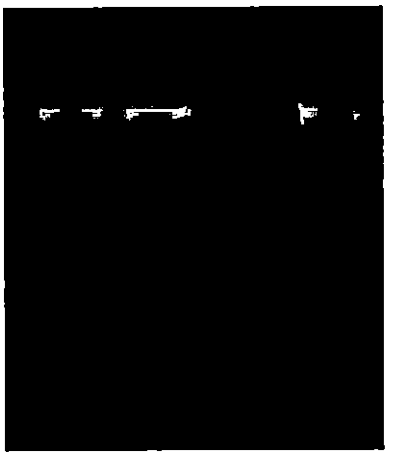

Plate 17. Genomic DNA of *Vanilla planifolia* isolated using Doyle and Doyle method of DNA isolation

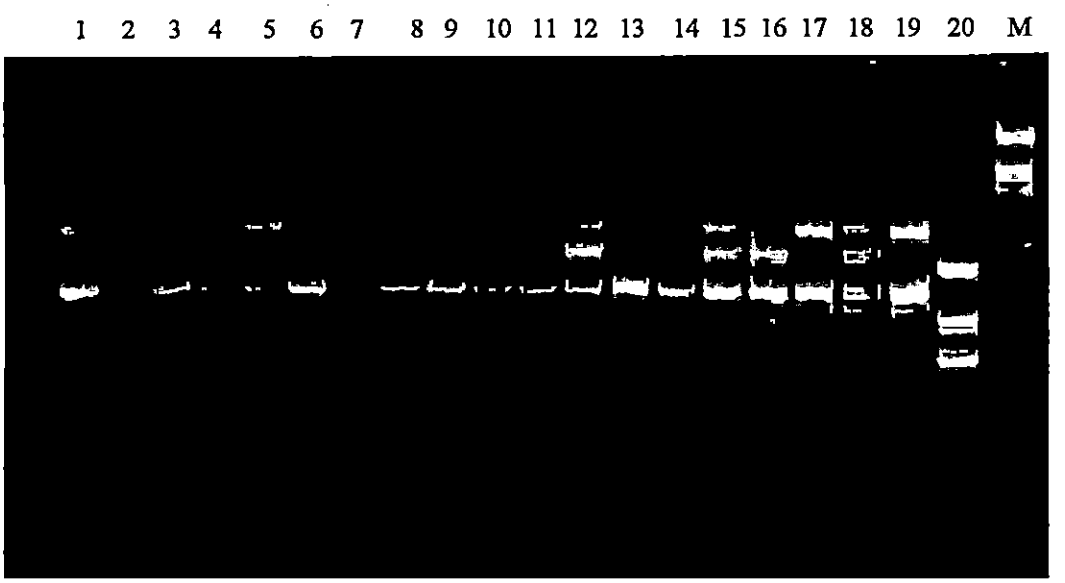

Plate 18. Amplification pattern shown by 20 different clones of vanilla using OPF 3 primer

Lanes are in the order of 1. a425, 2. a136, 3. a439, 4. a159, 5. a520, 6. a448, 7. a46, 8. a453, 9. a454, 10. a446, 11. a. 47, 12. a481, 13. a427, 14. a58, 15. a82, 16. a428, 17. a396, 18. a370, 19. a.555, 20. a468

4.3.2 RAPD assay

Perfect amplification was obtained with 50 ng template DNA, primer concentration of 5p moles and dNTPs at 200 μM levels, with 0.6 units enzyme. Intensity of bands was less at lower concentration of enzyme, primer and dNTPs. The primer concentration more than 5p moles resulted in primer-primer amplification. Minimum number of bands observed were two and maximum were eleven. The above mentioned combination of reaction mixture was found optimum for DNA amplification in vanilla. There was no amplification in the negative control samples (with out DNA) tried. For further studies, 50 ng template DNA was taken in all cases. The thermal cycle followed was optimum.

4.3.3 Screening of random primers

Twenty random primers were screened with the selected reaction mixture and thermal settings.

OPE series

Table 10 shows the results of screening of 18 primers of OPE series. Out of these OPE-3, 4, 7, 12, 14, 15, 18 and 20 gave good amplification. The primers OPE-14, 15 and 20 gave good amplification with five, four and five distinct bands respectively. Number of bands among the primers tested varied from zero to 5. OPE-3, 4, 7, 12, 14, 15, 18 and 20 were selected for further analysis.

OPF series

Both gave good amplification with 5 and 10 bands respectively. Subsequent trials gave the same results. OPF-2 and OPF-3 were those which were selected due to better stability for further analysis (Table 11).

The primers for further analysis were tested based on the number of bands, quality of amplification and stability of expression. Those primers which gave more distinct banding pattern with good quality amplification and reproducibility were selected for further analysis.

Table 9. Quantity and quality of genomic DNA isolated from Vanila

Sl. No.	Method of DNA isolation	Nature of bands	Absorbance		260/280 ratio	Qty (mg/g)	Quality
			at 260 nm	at 280 nm			
1	Doyle & Doyle method	Clear narrow	0.096	0.053	1.811	48	Very good

Table 10. Amplification pattern of vanilla genomic DNA with different decamer under OPE series at selected temperature profile and reaction mixture

Primer Code	Primer sequence	No. of bands	Quality of amplification
OPE 3	CCAGATGCAC	7	Good
OPE 4	GTGACATGCC	10	Good
OPE 5	GCAGGGAGGT	0	Poor
OPE 6	AAGACCCCTC	2	Average
OPE 7	AGATGCAGCC	9	Good
OPE 8	TCACCACGGT	0	Poor
OPE 9	CTTCACCCGA	2	Average
OPE 10	CACCAGGTGA	0	Poor
OPE 11	GAGTCTCAGG	3	Average
OPE 12	TTATCGCCCC	4	Good
OPE 13	CCCGATTCGG	0	Poor
OPE 14	TGCGGCTGAG	10	Good
OPE 15	ACGCACAACC	11	Good
OPE 16	GGTGACTGTG	3	Average
OPE 17	CTACTGCCGT	2	Average
OPE 18	GGACTGCAGA	10	Good
OPE 19	ACGGCGTATG	0	Poor
OPE 20	AACGGTGACC	10	Good

4.3.5 Amplification pattern

Out of the twenty different primers from operon series evaluated, ten primers which gave good polymorphism were selected after repeating for three times. Primer OPF-3 showed the highest percent of polymorphism. Least polymorphism was shown by OPE-12. Amplification patterns with the primers OPF-3, OPE-15 and OPE-20 are shown in the Plates 18, 19, 20 and 21 respectively. The details of amplification by each of the selected primer is given in Table 12.

Primer OPF-3

Altogether 10 amplified products were found out of which 9 were polymorphic. Band 4 was found to be monomorphic.

Primer OPF-2

OPF-2 primer produced 5 amplification products out of which none were monomorphic.

Primer OPE-3

The primer OPE-3 produced 3 amplification products out of which one was monomorphic and the other two were polymorphic. The first two bands were polymorphic and the third one was polymorphic.

Primer OPE-4

Seven amplification products were obtained by the primer OPE-4. Out of which 2 were monomorphic and 5 were polymorphic. Bands, 4 and 6 were monomorphic and bands 1, 2, 3, 5 and 7 were polymorphic.

Primer OPE-7

A total of 5 amplified products were produced. Bands 1, 3, 4 and 5 are polymorphic and band 2 is monomorphic.

Table 11. Amplification pattern of vanilla genomic DNA with different decamer primers under OPF series selected temperature profiles and reaction mixture

Primer code	Primer sequence	No. of bands	Quality of amplification
OPF 2	GAGGATCCCT	5	Good
OPF 3	CCTGATCACC	10	Good

Table 12. Amplicons obtained using 10 different decamer primers in seedling derived clones of vanilla

Sl.No.	Primer	Number of amplicons		Total
		Polymorphic	Monomorphic	
1	OPF-3	9	1	10
2	OPF-2	5	-	5
3	OPE-3	2	1	3
4	OPE-4	5	2	7
5	OPE-7	3	2	5
6	OPE-12	-	2	2
7	OPE-14	7	4	11
8	OPE-15	10	6	16
9	OPE-18	4	6	10
10	OPE-20	5	5	10

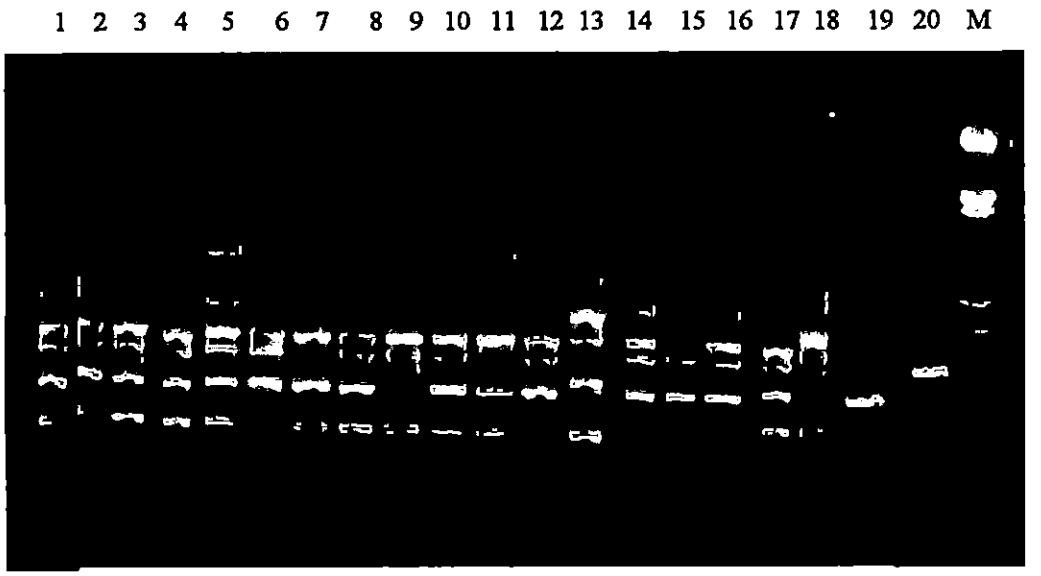

Plate 19. Amplification pattern shown by 20 different clones of vanilla using OPE 15 primer

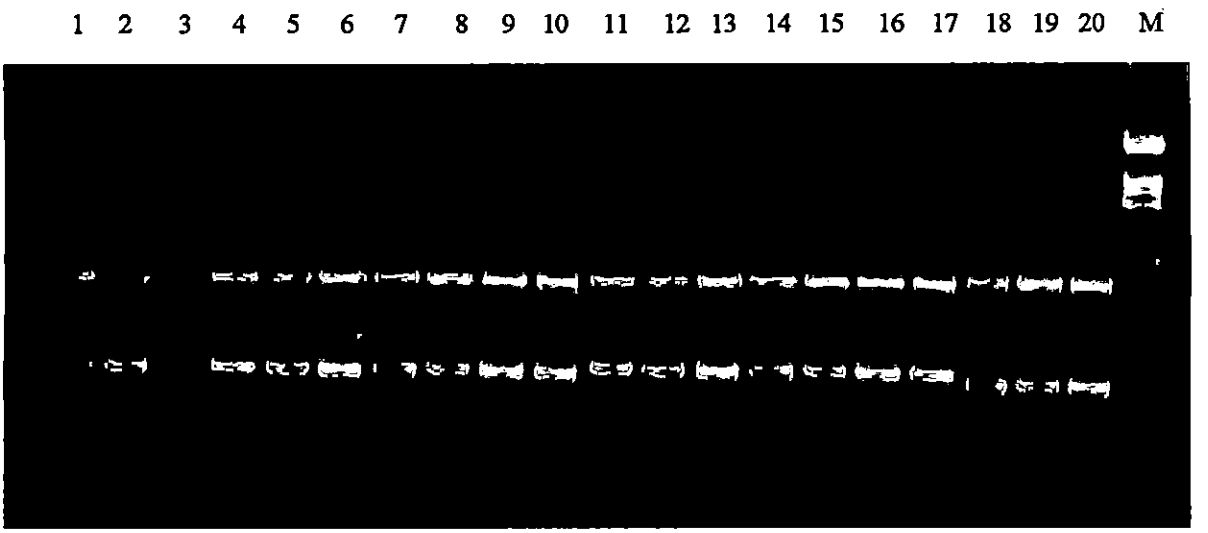

Plate 20. Amplification pattern shown by 20 different clones of vanilla using OPE 18 primer

Lanes are in the order of 1. a425, 2. a136, 3. a439, 4. a159, 5. a520, 6. a448, 7. a46, 8. a453, 9. a454, 10. a446, 11. a. 47, 12. a481, 13. a427, 14. a58, 15. a82, 16. a428, 17. a396, 18. a370, 19. a.555, 20. a468

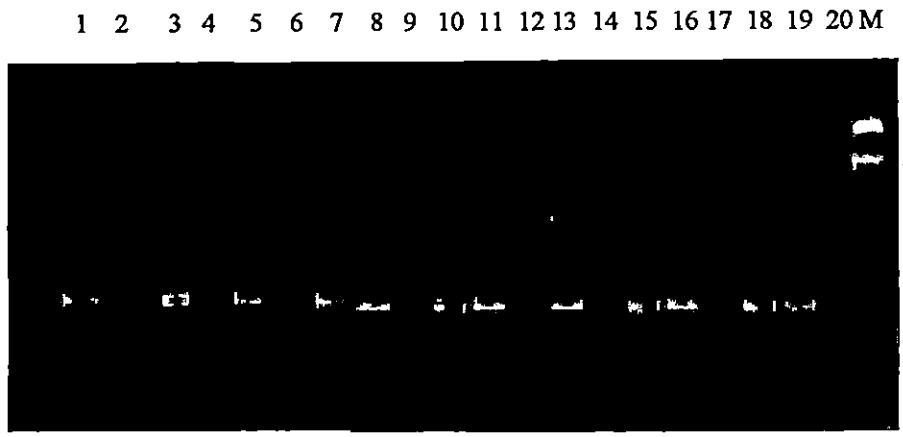

Plate 21. Amplification pattern shown by 20 different clones of vanilla using OPE 20 primer

Lanes are in the order of 1. a425, 2. a136, 3. a439, 4. a159, 5. a520, 6. a448, 7. a46, 8. a453, 9. a454, 10. a446, 11. a. 47, 12. a481, 13. a427, 14. a58, 15. a82, 16. a428, 17. a396, 18. a370, 19. a.555, 20. a468

Primer OPE-12

Only 2 amplicons were observed and both were monomorphic.

Primer OPE-14

A total of 11 amplified products were produced out of which 7 were polymorphic. Bands 1, 2, 6 and 9 were monomorphic.

Primer OPE-15

Sixteen amplification products were obtained by using this primer. Out of the 16 bands obtained, 6 were monomorphic and the remaining 10 were polymorphic. The bands 5, 8, 9, 10, 11 and 14 were monomorphic and the others were polymorphic. The bands 15 and 16 were distinct and clear for a47. The band 7 was distinct for a481.

Primer OPE-18

A total of 10 amplified products were produced out of which 6 were monomorphic. The band 5 and 8 were absent in a481. Band 2 was specific for a481.

Primer OPE-20

A total of 10 amplified products were produced out of which 5 were polymorphic. Band 1, 2, 4, 5 and 7 were monomorphic. Band 6 was absent in a427 and a370. Band 2 was present only in a481. Band 1 was found only in a46 and a481.

4.3.6 Cluster Analysis

The dendrogram constructed from the pooled data of the RAPD scores with 10 different primers expressed great variability in the genetic make up of vanilla plants evaluated (Fig. 5). The variation recorded ranged between 12 per cent to 55 per cent forming three major clusters. The first cluster included all the 13 clones with a total variability of around 30 per cent. The two clones a439 and a481 were very distinct but with maximum variability (50-55%). The clones a454 and a446 showed 88 per cent similarity in RAPD pattern. Another pair of clones (a82 and a58) also showed less than 20 per cent variability. All the other 16 clones indicated significant genetic variability in RAPD assay.

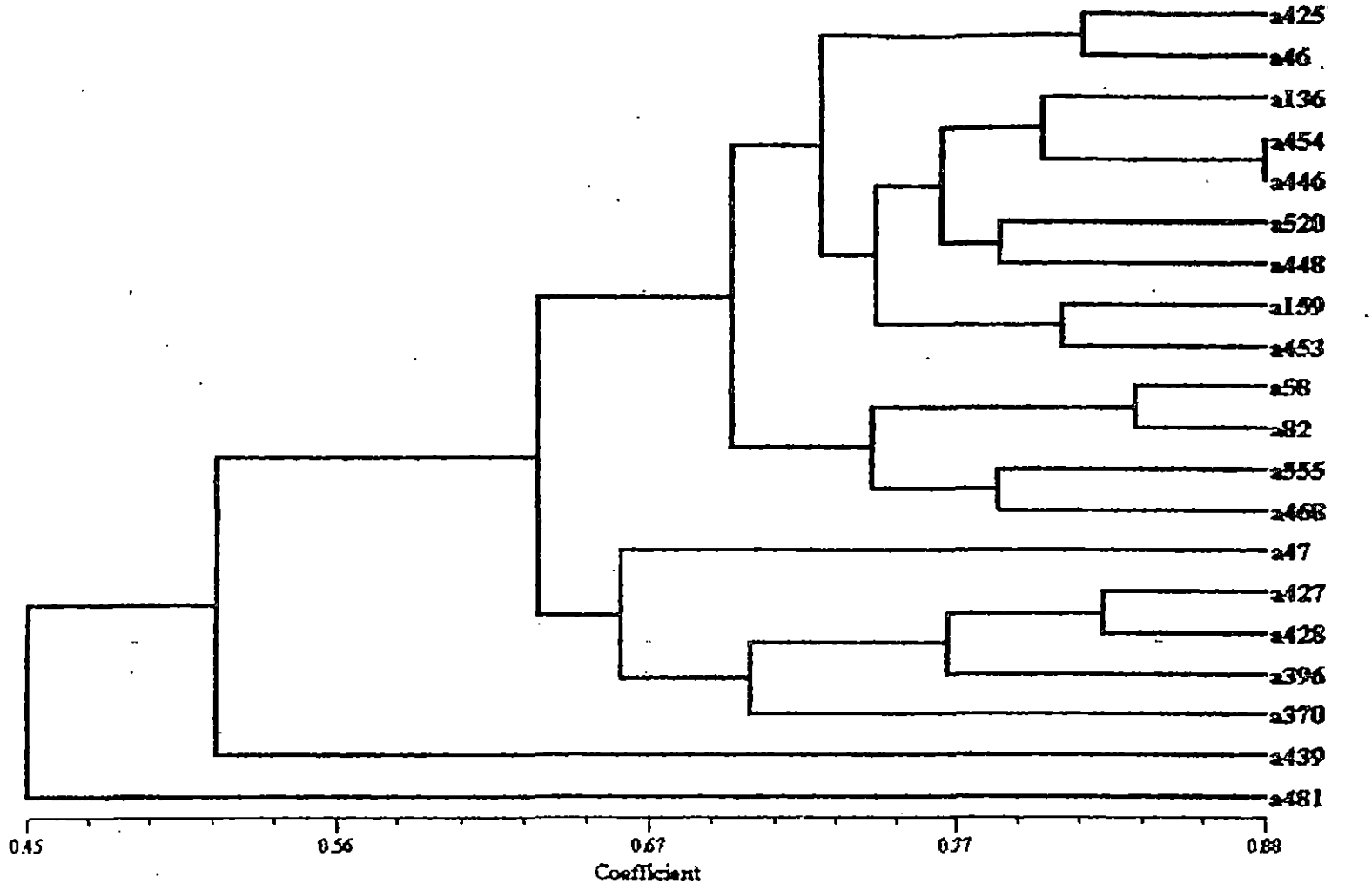

Fig. 5. Dendrogram showing the different clusters formed by the 20 different clones of vanilla for after RAPD analysis

Discussion

5. DISCUSSION

Vanilla planifolia Andrews. is a high valued exotic spice crop of India propagated exclusively through vegetative means. Being an orchid, the plant lack normal seeds and the viable naked embryos always require a nourishing medium for germination and further existence. Thus the natural variability in vanilla through seed population is highly limited. Large extent of area was brought under vanilla culture owing to the high price for the harvested pods. Efforts are being made to induce variability in vanilla through various techniques and thus to increase diversity for crop improvement. The present study was taken up to evaluate the extent of variability induced in vanilla through *in vitro* techniques. Morphological, biochemical and molecular characterizations were taken up to evaluate the plants.

5.1 MORPHOLOGICAL MARKERS

Wide variations in morphological characters were observed in the different accessions studied.

The 360 plants studied under 72 accessions exhibited variation for leaf characters namely,leaf length, leaf width and leaf area (Table 1).

Leaf length ranged from 3.52 cm to 14.90 cm and leaf width ranged from 2.10 cm to 4.00 cm. The leaf area was found to vary widely in the accessions and ranged from 3.55 cm^2 to 41.23 cm^2. In all the accessions studied, the leaves were alternate and was oblong-elliptic in shape. The intraclonal variation recorded for tissue culture derived vanilla was insignificant, while it was significant in clones derived from different seeds in the same pod and also from different pods.

As observed in the present study, variation for leaf morphology was reported by various workers in micropropagated plants of different crop species including vanilla. Mathur *et al.* (1988) reported extensive somaclonal variation in callus derived plants of Java citronella for length and area of the longest leaf. Similarly, Israeli *et al.* (1991) observed variations in leaf morphology of

micropropagated field planted bananas of Cavendish and Red sub groups. Variability has been observed in the seedling of vanilla especially in their leaf morphology and phyllotaxy (Mary *et al.*, 1999). Similar variations on morphological characters were also reported in *in vitro* regenerants of wheat (Hashim *et al.*, 1988), sorghum (Maralappanavan *et al.*, 1995), oat (Dahleen *et al.*, 1995), pigeon pea (Prasannalatha *et al.*, 1994) and safflower (Seeta and Anwar, 1992).

The number of leaves and the total growth in the vanilla accessions studied varied from 21 to 87 leaves and 97 cm to 695 cm respectively (Table 2). The internodal length ranged from 2.200 cm to 4.300 cm (Table 1) and it varied widely among different accessions. The roots in the *in vitro* derived plants were normal. They originated opposite to the leaves and were single. Genetic variability in vanilla seedlings raised through ovule culture has been reported earlier after studying the various morphological parameters like leaf size, internodal length, growth rate and isozyme pattern (Divakaran *et al.*, 1996). The present study also confirmed the variability in *in vitro* derived seedling clones of vanilla. Stable variation was observed for leaf size, growth rate and internodal length. The clones a82, a425, a58 and a136 were found to be vegetatively more vigorous from others.

Since the vanillery had not started flowering and fruiting during the time of observation, floral and pod characters were not recorded.

5.2 ISOZYME VARIATIONS

5.2.1 Protein estimation

Quantitative estimation of protein was carried out using Bradford's method. The quantity of the protein varied from 0.063 mg ml^{-1} to 0.552 mg ml^{-1} extract. Comparison with the banding pattern of isozymes of the same samples revealed that protein is independent of the enzymes studied. Therefore, the protein content is independent of the factor/enzyme studied, which requires further screening and analytical approaches to exploit for this type of work. The results of protein estimation are in line with the reports of McCoy (1979) and Swaminathan (2002) recorded variations in the protein concentrations of different crop species.

The tissue culture derived vanilla clones showing high morphological variability were evaluated for two isozymes namely, esterase and peroxidase. Leaves from different positions were used as the source material in the study and it was observed that the third open leaf from the growing tip was the ideal leaf sample for isozyme assay in vanilla. Both the isozyme studied showed significant variation among the 20 different accessions. Out of the 10 isozyme bands observed for esterase, only two were found monomorphic for all the accessions studied indicating the variability among the clones studied. Some of the bands (EST 24, EST 30 and EST 38) were shared by more than three individuals while a few others (EST 43 and EST 70) were found specific to clones a370 and a425 respectively.

The isozyme assay for peroxidase also showed a similar pattern and only three bands out of 12 were found monomorphic to all the accessions studied. Some of the bands like PRX 10 was found unique to accession a159, while PRX 43, PRX 17 and PRX 24 were found always shared by more than four clones.

It was interesting to note that the clone a82 and a425 were unique from others with respect to peroxidase and esterase activity. However a82 was found associated with clone a555 in peroxidase assay. These clones were screened after morphological characterisation owing to their better vegetative growth compared to other accessions. The unique banding pattern showed by these clones strengthen the views for linking the phenotypic characters with biochemical markers. Further studies in this line involving more plants would help to identify biochemical markers for early detection of vigorous plants at hardening stage itself. The esterase bands, EST 18, EST 20, EST 70 and peroxidase band PRX 40 can be further exploited for developing biochemical markers in vanilla.

Considering variation in the banding pattern of both the enzymes in different accessions, it can be deduced that isozyme polymorphism could reliably be used to characterise the accessions in *Vanilla planifolia*.

Isozyme characterization was done successfully in pepper, cassava, curcuma, arecanut and in many other horticultural crops in order to identify the cultivars and to characterize the genotypes biochemically. Differences in the banding patterns in the form of presence of an additional band or absence of a common band could be correlated with disease resistance / tolerance as reported by Deyu (1995) in barley, Gupta *et al.* (1995) in brassica and Fei *et al.* (1997) in soybean.

To sum up, the isozyme banding pattern revealed the presence of considerable variability in the vanilla accessions. The most dissimilar accessions were a425, a58, a481 and a370. The study also indicated few isozyme markers linked with better vegetative growth in vanilla that could be explained further.

5.3 DNA MARKERS

Molecular markers have been proved as a fundamental and reliable tool for fingerprinting varieties, establishing the fidelity of progenies etc. The advent of automated PCR (Polymerase Chain Reaction) technology made a new set of markers available to scientists interested in comparing organisms at molecular level. Williams *et al.* (1990) were the first to use random amplified polymorphic DNA markers, obtained by PCR amplification of DNA segments with single arbitrary primers.

The random amplified polymorphic DNA (RAPD) reaction performed on genomic DNA with an arbitrary oligonucleotide results in the amplification of reveal discrete DNA products. They are usually separated on agarose gel and visualised by ethidium bromide staining. The polymorphism between individuals result from sequence difference in one or both of the primer binding sites and are visible as presence or absence of a particular band. Such polymorphism, in general, behave as dominant genetic markers. The RAPD amplification generated can be classified into two types: constant (monomorphic) and variable (polymorphic). These differences can be used to examine and establish systematic relationship (Hadrys *et al.*, 1992).

5.3.1 DNA isolation

In this study, Doyle and Doyle (1987) method of DNA isolation was effective in vanilla and gave sufficient quantity of DNA with good quality. Since the

procedure did not require liquid nitrogen, DNA isolation was more economic also. The quantity of DNA recovered was proportionate with the amount of leaf sample used for isolation. However, the DNA was found degraded whenever the amount of leaf sample was increased.

The chloroform isoamyl alcohol treatment was found to give good quality, DNA with less impurity. The usefulness of chloroform isoamyl alcohol treatment to precipitate protein and improve the quality of DNA of black pepper was reported by Babu (1997). The RNase treatment was found effective in order to remove RNA from the DNA sample and the quality and purity of the sample improved with the treatment. This is in line with the reports of Babu (1997) and Mondal *et al.* (2000). Since one RAPD reaction mixture required 0.25 to 50 mg of DNA, the DNA recovered in this method was found sufficient for further RAPD analysis.

5.3.2 RAPD analysis

The RAPD technique in vanilla was carried out using genomic DNA from 20 different clones of vanilla. The reaction mixture with primer concentration of 5 p moles and dNTPs at 100µM levels with 0.6 units enzyme was found optimum for perfect amplification. 50ng template DNA was taken for the assay. The thermal cycle followed was ideal and gave good amplification.

Twenty operon random decamer primers from OPE and OPF series were used for screening. All the primers did not amplify well. From the twenty primers used for screening, ten primers were finally selected for RAPD analysis of vanilla accessions. The selected primers were OPE 3, 4, 7, 12, 14, 15, 18 and 20 and OPF 2 and 3. Two to eleven bands were observed in the RAPD profile for each primer. Primer OPF-3 showed the highest per cent of polymorphism and least polymorphism was shown by OPE 12.

This study points out that accessions under study are divergent with respect to RAPD markers. The extent of variability was comparable to the morphological and biochemical markers. Deviations in conventional taxonomic relationship based on

DNA data were reported earlier also (Chakrabarti *et al.*, 1999; Padmesh *et al.*, 1999). Williams *et al.* (1990) had reported that RAPD loci are likely to be found within non - coding regions or not being closely linked to structural genes. This is probably the reason for differences observed sometimes between conventional markers like morphological and isozyme markers on one hand and DNA markers on the other. Phenotypic markers cannot identify the genetic differences of non-coding genes. He also attributed polymorphism between genotyes to nucleotide changes that prevent amplification by introducing a mismatch at one primary site, deletion of a priming site, insertions that render priming sites too distant to support amplification and insertions or deletions that change the size of the amplified product. Therefore, these differences between the vanilla accessions would lead to the polymorphism in the RAPD analysis. Such polymorphism make RAPD well suited for studies on genetic diversity and genetic relationships.

Similarity matrix derived from RAPD scores was subjected to Sequential Agglomerative Heirarchial Nested Clustering (SAHN). A phenetic dendrogram was constructed using Unweighted Pair Group Method of Arithematic Averages (UPGMA) by Sneath and Sokel (1973) using NTSYS package. As can be seen from Fig.5 three major clusters were obtained from the pooled data of ten primers. Accessions, a454 and a446 were found genotypically closer. Similarly a58 and a82, a425 and a46, a520 and a448, a159 and a453, a555 and a468, a427 and a428 were found genotypically closer with 88 percent similarity. The clones a82 and a58 also showed less than 20 percent variability. The clones a481, a439, a370, a47, a396, a136, a425, a46, a520, a448, a159, a453, a555, a468, a427 and a428 were found to be genetically diverse.

Thus, the present study confirmed genetic variability in seedling derived clones of vanilla through morphological, biochemical and molecular assay. Further more, the results could highlight the presence of biochemical and molecular markers linked to better vegetative growth in vanilla clones. Distinct isozyme and RAPD bands were found linked with better performing clones like a82, a58 and a425. This can be further exploited after evaluating the yield performance of these clones.

Summary

6. SUMMARY

The present study "Morphological and molecular characterization of variability in *in vitro* derived seedlings of vanilla (*Vanilla planifolia* Andrews) was conducted in the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the year 2001-2003.

The study intended to characterise 360 vanilla accessions based on morphological markers and then to further evaluate 20 selected accessions from it and characterise those based on molecular markers.

Morphological data on the 10 characters for the 360 accessions were analysed statistically. The analysis revealed that all the accessions differed significantly for 6 characters, viz., leaf length, leaf width, leaf area, number of leaves, total growth and internodal length. In all the clones studied, the four characters viz., leaf shape, phyllotaxy, root origin and number of roots were similar. The floral and bean characters were not recorded due to the absence of flowering during the period of observation. From the 360 accessions studied, 20 different accessions from different pods were selected for further biochemical and molecular analysis.

Biochemical studies based on isozyme markers were carried out in the selected 20 accessions. The protocol for isozyme analysis could be standardised. The isozymes studied were esterase and peroxidase. Isozyme studies could group the 20 different accessions into different clusters based on the dendrogram constructed. The accessions were grouped into nine clusters based on esterase analysis and into 15 groups based on peroxidase analysis. Based on esterase analysis accessions a82, a481, a468, a425, a396, a58 and a370 were found highly polymorphic.

Molecular studies using RAPD (Random Amplified Polymorphic DNA) markers were carried out for the 20 different accessions. The DNA isolation protocol was standardised by slightly modifying the Doyle and Doyle (1987) method and then the RAPD analysis was done with 10 selected random decamer primers. The 20 accessions under study were found divergent with respect to RAPD markers. Based on

the dendrogram constructed, the 20 accessions were grouped into 3 major clusters with 55 per cent variability. Accessions a454 and a446 showed maximum similarity (12%).

The study confirmed uniformity among *in vitro* derived vanilla plants and the variability generated through *in vitro* seed culture. The tissue culture derived plants of same clones were uniform with respect to all the morphological characters studied. High variability was observed for plants derived from different seeds in the same pod and from different pods. Few isozyme markers were found linked with better vegetative growth (EST 70 and EST 43; PRX 40 and PRX 10). The RAPD analysis also confirmed genetic variability in the accessions studied.

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* Originals not seen

Annexures

Annexure-1

Details of vanilla clones selected for morphological evaluation

Pod No.	Clone No.	Accession No.
VP47	a 275	1.1.1 1.1.2 1.1.3 1.1.4 1.1.5
	a 331	1.2.1 1.2.2 1.2.3 1.2.4 1.2.5
	a 332	1.3.1 1.3.2 1.3.4 1.3.4 1.3.5
	a 94	1.4.1 1.4.2 1.4.3 1.4.4 1.4.5
	a 106	1.5.1 1.5.2 1.5.3 1.5.4 1.5.5
VP67	a 2	2.1.1 2.1.2 2.1.3 2.1.4 2.1.5
	a 46	2.2.1 2.2.2 2.2.3 2.2.4 2.2.5
	a 47	2.3.1 2.3.2 2.3.3 2.3.4 2.3.5
	a 58	2.4.1 2.4.2 2.4.3 2.4.4 2.4.5

	a 32	2.5.1 2.5.2 2.5.3 2.5.4 2.5.5
	a 13	2.6.1 2.6.2 2.6.3 2.6.4 2.6.5
VP x 3	a 436	3.1.1 3.1.2 3.1.3 3.1.4 3.1.5
	a 425	3.2.1 3.2.2 3.2.3 3.2.4 3.2.5
	a 427	3.3.1 3.3.2 3.3.3 3.3.4 3.3.5
	a 430	3.4.1 3.4.2 3.4.3 3.4.4 3.4.5
	a 428	3.5.1 3.5.2 3.5.3 3.5.4 3.5.5
	a 434	3.6.1 3.6.2 3.6.3 3.6.4 3.6.5
VP x 4	a 439	4.1.1 4.1.2 4.1.3 4.1.4 4.1.5
	a 451	4.2.1 4.2.2 4.2.3 4.2.4 4.2.5

	a 454	4.3.1 4.3.2 4.3.3 4.3.4 4.3.5
	a 457	4.4.1 4.4.2 4.4.3 4.4.4 4.4.5
	a 446	4.5.1 4.5.2 4.5.3 4.5.4 4.5.5
	a 453	4.6.1 4.6.2 4.6.3 4.6.4 4.6.5
VP 91	a 136	5.1.1 5.1.2 5.1.3 5.1.4 5.1.5
	a 159	5.2.1 5.2.2 5.2.3 5.2.4 5.2.5
	a 137	5.3.1 5.3.2 5.3.3 5.3.4 5.3.5
	a 139	5.4.1 5.4.2 5.4.3 5.4.4 5.4.5
	a 204	5.5.1 5.5.2 5.5.3 5.5.4 5.5.5
VP 229	a 962	6.1.1 6.1.2 6.1.3 6.1.4 6.1.5

	a 866	6.2.1 6.2.2 6.2.3 6.2.4 6.2.5
	a 861	6.3.1 6.3.2 6.3.3 6.3.4 6.3.5
	a 712	6.4.1 6.4.2 6.4.3 6.4.4 6.4.5
	a 710	6.5.1 6.5.2 6.5.3 6.5.4 6.5.5
VP 70	a 369	7.1.1 7.12 7.1.3 7.1.4 7.1.5
	a 362	7.2.1 7.22 7.2.3 7.2.4 7.2.5
	a 410	7.3.1 7.32 7.3.3 7.3.4 7.3.5
	a 396	7.4.1 7.42 7.4.3 7.4.4 7.4.5
	a 370	7.5.1 7.52 7.5.3 7.5.4 7.5.5
VP x 1	a 468	8.1.1 8.1.2 8.1.3 8.1.4 8.1.5

	a 481	8.2.1 8.2.2 8.2.3 8.2.4 8.2.5
	a 520	8.3.1 8.3.2 8.3.3 8.3.4 8.3.5
	a 475	8.4.1 8.4.2 8.4.3 8.4.4 8.4.5
	a 489	8.5.1 8.5.2 8.5.3 8.5.4 8.5.5
	a 448	8.6.1 8.6.2 8.6.3 8.6.4 8.6.5
VP 229	a 580	9.1.1 9.1.2 9.1.3 9.1.4 9.1.5
	a 856	9.2.1 9.2.2 9.2.3 9.2.4 9.2.5
	a 720	9.3.1 9.3.2 9.3.3 9.3.4 9.3.5
	a 777	9.4.1 9.4.2 9.4.3 9.4.4 9.4.5
	a 555	9.5.1 9.5.2 9.5.3 9.5.4 9.5.5

	a 544	9.6.1 9.6.2 9.6.3 9.6.4 9.6.5
VP 228	a 777	10.1.1 10.1.2 10.1.3 10.1.4 10.1.5
	a 820	10.2.1 10.2.2 10.2.3 10.2.4 10.2.5
	a 803	10.3.1 10.3.2 10.3.3 10.3.4 10.3.5
	a 854	10.4.1 10.4.2 10.4.3 10.4.4 10.4.5
	a 887	10.5.1 10.5.2 10.5.3 10.5.4 10.5.5
VP 46	a 81	11.1.1 11.1.2 11.1.3 11.1.4 11.1.5
	a 83	11.2.1 11.2.2 11.2.3 11.2.4 11.2.5
	a 84	11.3.1 11.3.2 11.3.3 11.3.4 11.3.5
	a 79	11.4.1 11.4.2 11.4.3 11.4.4 11.4.5

	a 82	11.5.1 11.5.2 11.5.3 11.5.4 11.5.5
VP 156	a 307	12.1.1 12.1.2 12.1.3 12.1.4 12.1.5
VP 229	a 648	13.1.1 13.1.2 13.1.3 13.1.4 13.1.5
VP 90	a 128	14.1.1 14.1.2 14.1.3 14.1.4 14.1.5
VP 47	a 281	15.1.1 15.1.2 15.1.3 15.1.4 15.1.5
VP 156	a 305	16.1.1 16.1.2 16.1.3 16.1.4 16.1.5
VP 155	a 341	17.1.1 17.1.2 17.1.3 17.1.4 17.1.5
VP 230	a 696	18.1.1 18.1.2 18.1.3 18.1.4 18.1.5
VP 230	a 698	19.1.1 19.1.2 19.1.3 19.1.4 19.1.5
VP 155	a 346	20.1.1 20.1.2 20.1.3 20.1.4 20.1.5

VP 159	a 356	21.1.1 21.1.2 21.1.3 21.1.4 21.1.5
VP 153	a 349	22.1.1 22.1.2 22.1.3 22.1.4 22.1.5
VP 52	a 70	23.1.1 23.1.2 23.1.3 23.1.4 23.1.5

Annexure-2

Details of clones selected for biochemical and molecular evaluation

Sl. No.	Pod No.	Clones
1	VP46	a82
2	VP1	a520
3	VP3	a427
4	VP3	a428
5	VP91	a159
6	VP4	a47
7	VP9	a46
8	VP91	a136
9	VP229	a555
10	VP70	a396
11	VP67	a58
12	VP70	a370
13	VP4	a439
14	VP4	a446
15	VP4	a448
16	VP4	a453
17	VP4	a454
18	VP1	a481
19	VP1	a468
20	VP3	a425

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF VARIABILITY IN *IN VITRO* DERIVED SEEDLINGS OF VANILLA (*Vanilla planifolia* Andrews)

By

K. K. HENA

ABSTRACT OF THE THESIS

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Kerala Agricultural University

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ABSTRACT

The research project "Morphological and molecular characterization of variability in *in vitro* derived seedlings of vanilla (*Vanilla planifolia* Andrews)" was carried out at the College of Horticulture, Vellanikkara, Thrissur during the period 2001-2003. The major objectives of the study was to characterize the existing variability in field established vanilla plants, derived from *in vitro* seed culture, using morphological and molecular markers.

The study revealed that among the 10 morphological characters selected i.e., leaf length, leaf width, leaf area, number of leaves, total growth, leaf shape, phyllotaxy, root origin and number of roots, except leaf shape, phyllotaxy, root origin and number of roots, all other characters showed significant variation. Twenty different accessions which were highly variable could be selected from the 360 accessions studied.

In biochemical studies, the protocol for isozyme analysis could be standardised. The study was done based on esterase and peroxidase patterns. Based on the dendrogram constructed, the 20 different accessions studied could be grouped into different clusters. Based on esterase pattern they were grouped into 9 clusters and based on peroxidase pattern they were grouped into 15 clusters. The accessions a82, a481, a58, a370 and a425 showed high level of polymorphism in both the isozyme patterns. Accessions a82, a396, a370 and a425 showed high level of polymorphism in esterase pattern and accessions a555, a136, a46, a428, a47 a159and a425 showed polymorphism in peroxidase pattern.

Molecular studies involved RAPD analysis using 10 primers which gave 16 amplification products. Dendrogram constructed based on the study grouped the 20 accessions into 3 major clusters. The 20 different accessions studied were varying from each other in both the morphological and molecular studies. Hence, it can be said that there is considerable variability in the *in vitro* derived seedlings of vanilla.

The study revealed a similar trend for morphological and molecular markers in assessing variability. Morphological markers need more refinement so as to get precise information on the yield characters too which is more important in case of vanilla. Molecular studies based on AFLP, RFLP etc. can be attempted to get exact picture on variability in vanilla.